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(6) LABORATORY EVALUATION OF THE TOXICITY OF CYCLOTRIMETHYLENE TRINITRAMINE (RDX) TO AQUATIC ORGANISMS

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The toxicity of nitroglycerine to a wide variety of aquatic organisms representing several different trophic levels in aquatic ecosystems was studied. Results of static acute toxicity tests indicate that the acute LC50 values are greater than 3 mg/l RDX. There was an apparent lack of bioaccumulation in edible or nonedible tissues or organs in all species tested. Effects were observed on growth at 5.8 mg/l RDX during egg and fry studies, on survival at 4.9-6.3 mg/l during chronic exposure of fathead minnows, and on number of young produced per parthenogenetic female at concentrations ≥ 4.8 mg/l. Applying an application factor of 0.1 to the lower limit of observed acute toxicity → equal to or greater than

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20. values (3.6 mg/l, 96-hour LC50 for bluegill at pH 6.0) a water quality criterion of 0.35 mg/l RDX is proposed.

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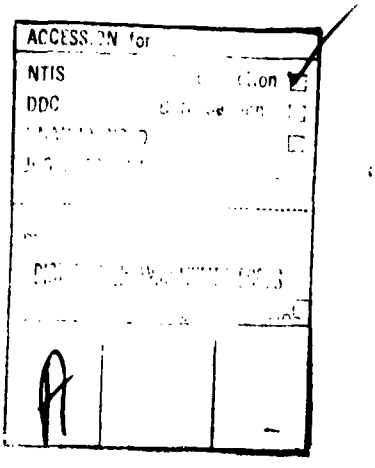

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SUMMARY

In order to provide the data base required to perform a hazard evaluation relative to the occurrence of RDX (cyclotrimethylene trinitramine) in the aquatic environment and to recommend a proposed water quality criterion for RDX for the protection of freshwater aquatic life, acute, subacute and chronic toxicological evaluations were performed on a wide variety of freshwater organisms representing several trophic forms utilizing RDX. Additionally, the bioaccumulation of 14C-RDX in fishes was investigated.

The results of static acute toxicity tests indicate that the acute LC50 values generally are greater than 3 mg/l RDX. RDX exhibited essentially no toxicity to the four species of algae and the four species of invertebrates during static acute toxicity tests. The calculated LC50 values for all fish species ranged from 4.1 to 6.0 mg/l during static acute toxicity tests. In a comparison between the toxicity of RDX observed in static versus flow-through testing, RDX exhibited essentially no difference for either fishes or invertebrates.

The results of the bioaccumulation study with 14C-RDX indicate an apparent lack of appreciable accumulation in the edible or nonedible tissues or organs of all species tested. Effects on growth of critical life stages of the fathead minnow were observed

at 5.8 mg/l, and on survival at 4.9 - 6.3 mg/l during chronic exposure of fathead minnows over one life cycle. Midges exposed chronically to RDX appeared unaffected by concentrations as high as 21 mg/l, while in the daphnid chronic exposure, the number of young produced per parthenogenetic female was effected at concentrations $\geq$4.8 mg/l.

Based on fathead minnow chronic and acute toxicity data, the limits on an application factor specific for RDX are >2.7/5.2 and <6.3/5.2 or >0.52 and <1.2. In view of these data it appears that applying an application factor of 0.1 to the acute toxicity data would adequately protect aquatic life during chronic exposure. Applying this application factor to the lower limit of observed acute toxicity values,(3.6 mg/l, 96-hour LC50 for bluegill at pH 6.0, 20^{o}C) a water quality criterion of 0.35 mg/l RDX is proposed for the protection of freshwater aquatic life with an adequate margin of safety.

INTRODUCTION

Cyclotrimethylene-trinitramine (RDX) is known to occur in discharges from the Holston Army Ammunition Plant in Kingsport, Tennessee and in numerous load and pack operations. Water and Air Research (1976) projected the discharge of RDX from any proposed facilities at <0.01-0.3 mg/l under normal operating conditions and under the worst operating conditions would not exceed 2.9 mg/l. Since there appears to be very little information on the toxicity of RDX on aquatic organisms, studies were initiated to investigate the acute and chronic toxicity of RDX on aquatic organisms. The objective of the program was to provide a portion of the data base required to perform a hazard evaluation relative to the occurrence of RDX in the aquatic environment, and to recommend a proposed water quality criterion for RDX for the protection of freshwater aquatic life with an ample margin of safety.

The specific efforts undertaken included investigations of: (a) the acute toxicity of RDX to a wide variety of aquatic organisms representing all trophic levels under static and flowing water (flow-through) conditions; (b) the effects of variations in water quality on the acute toxicity of RDX to fish; (c) the chronic toxicity of RDX to both aquatic invertebrate and vertebrate organisms; and (d) the bioconcentration of RDX by fishes.

The studies to evaluate the acute toxicity of RDX to phytoplankton were performed at the Marine Research Laboratory of E G & G,

Bionomics in Pensacola, Florida. The studies to evaluate the toxicity of RDX to all other aquatic organisms were conducted at the Aquatic Toxicology Laboratory of E G & G, Bionomics in Wareham, Massachusetts. Chemical analyses were performed at the Analytical Chemistry Laboratories of E G & G, Bionomics in Wareham, Massachusetts.

MATERIALS AND METHODS

Test Material

The production grade cyclotrimethylene trinitramine (RDX) used in these studies was obtained from the Holston Army Ammunition Plant in Kingsport, Tennessee and Aberdeen Proving Ground, Aberdeen, Maryland (Table 1). For all phytoplankton tests, the RDX was added directly to the test medium. For all static, acute toxicity tests, the RDX was dried and mixed with acetone to form a superstock solution. Due to miscibility problems of this stock solution, a more appropriate solvent was determined to be dimethyl sulfoxide (DMSO), which appeared to maintain more of the RDX in solution. Therefore, all subsequent testing was conducted with a DMSO/RDX superstock solution. Concentrations of RDX are reported as miligrams (mg) per liter (l) of diluent water, or parts per million (ppm).

The 14C-RDX used in the bioconcentration study was received in a vial from New England Nuclear, Boston, Massachusetts. Correspondence which accompanied the vial stated that it contained 2.00 millicuries (mCi) of 14C-cyclonite (synonym for RDX) in 130.7 mg of cyclonite (lot #846-216). The entire contents of this vial were transferred to a 100 ml volumetric flask and brought to volume with DMSO, and served as a 14C-RDX solution from which working stock solutions were prepared for this study.

Test Organisms

Algae tested were the cyanophytes (blue-greens) Microcystis aeruginosa and Anabeana flos-aquae; the chlorophyte (green) Selenastrum capricornutum; and the chrysophyte (diatom) Navicula pelliculosa. Cultures were obtained from the collection at the University of Indiana, Bloomington, Indiana, and the Pacific Northwest Water Quality Laboratory (EPA, Corvallis, Oregon). Each species was maintained in stock cultures at Bionomics Marine Research Laboratory. Culture medium was prepared according to the formula described in "Algal Assay Procedure: Bottle Test" (U.S. EPA, 1971a).

Macroinvertebrates exposed to RDX were the water flea (Daphnia magna), scud (Gammarus fasciatus), sowbug (Asellus militaris), and midge (Chironomus tentans). The water flea were acquired from Bionomics' laboratory cultures, and the scud, sowbug, and midge were collected in the Wareham, Massachusetts area by Bionomics' personnel.

At the initiation of testing, water flea were 0-24 hours old, scud and sowbug were in the juvenile stage, and the midge larvae were in the second or third instar stage.

Fish utilized in the acute, static toxicity tests were bluegill (Lepomis macrochirus), rainbow trout (Salmo gairdneri), channel catfish (Ictalurus punctatus), and fathead minnow (Pimephales

promelas). Unless otherwise noted, the bluegill were acquired from a commercial fish farmer in Nebraska, and had a mean (± S.D.) weight of 1.0 (± 0.3) g and a mean (± S.D.) standard length of 35 (± 6) mm. The rainbow trout were acquired from a commercial trout producer in Massachusetts and had a mean weight and length of 0.9 (± 0.3) g and 43 (± 4) mm, respectively. The channel catfish were obtained from a fish farmer in Arkansas, and had a mean weight of 1.2 (± 0.5) g and a mean length of 57 (± 11) mm. The fathead minnow were obtained from a commercial producer in Arkansas, and had a mean weight of 1.0 (± 0.4) g and a mean length of 43 (± 8) mm. For all tests, thirty fish representative of test populations of each species were weighed and measured for the calculation of means and standard deviations for each group.

Flow-through bioassays with RDX were conducted with bluegill obtained from a commercial hatchery in Connecticut and had a mean weight of 2.0 (± 0.2) g and mean standard length of 41 (± 3) mm; fathead minnows were acquired from a commercial hatchery in Arkansas and had a mean weight and length of 1.5 (± 0.2) g and 44 (± 4) mm, respectively; and channel catfish acquired from a commercial fish farmer in Missouri and had a mean weight and length of 2.1 (± 0.2) g and 45 (± 3) mm, respectively.

To investigate the bioaccumulation of 14C-RDX by fishes, bluegill, fathead minnows, channel catfish and rainbow trout were obtained from various commercial hatcheries. The mean weight and standard

lengths of the fishes utilized for these studies were: bluegill, 2.1 (± 0.3) g and 42 (± 4) mm; fathead minnows 2.3 (± 0.4) g and 59 (± 6) mm; channel catfish 1.4 (± 0.2) g and 46 (± 4) mm; and rainbow trout 1.2 (± 0.3) g and 48 (± 5) mm.

To investigate the chronic toxicity of RDX to fathead minnows, eggs were received from the EPA, Environmental Research Laboratory in Newtown, Ohio.

Prior to use in tests, all fish were held in 1700-l concrete raceways which were coated with an epoxy resin paint to prevent leaching of materials into the water. Flow of well water (temperature, 20 ± 1.0°C for bluegill, channel catfish, and fathead minnow, and 14 ± 1.0°C for the rainbow trout; hardness, 35 mg/l as $CaCO_3$; pH 7.1, and dissolved oxgyen concentration, >60% of saturation) into these raceways was at a minimum rate of 4 l/minute, which provided an adequate water turnover for holding these species. The fishes were maintained in these laboratory hatchery facilities for at least thirty days prior to use in bioassays. During this period, cumulative mortality for each species was <2%; no mortality was observed during the 48 hours immediately prior to testing, and these fishes were judged to be in excellent condition. Fish of each species were from the same year class, and the standard length of the longest fish was no more than twice that of the shortest fish.

Test Methods

(A) Static Acute Toxicity Tests - In order to evaluate the relative susceptibility of a broad spectrum of aquatic organisms to RDX, static bioassays were conducted.

During all bioassays to investigate the acute toxicity of RDX to aquatic organisms, two series of concentrations were established within each bioassay, a series of range-finding concentrations (preliminary test) and a series of definitive concentrations (definitive test). The preliminary test was conducted to determine an approximate range of concentrations for evaluating the dose-response relationship. The definitive test, consisting of at least five concentrations, evaluated the dose-response relationship to a degree allowing the median effective concentration (EC50) or the median lethal concentration (LC50) to be calculated from the data with optimum accuracy.

Algal assays were conducted according to the method described in "Algal Assay Procedure: Bottle Test" (U.S. EPA, 1971a). To determine the effects of RDX on algae, measurements were made of the chlorophyll a content of exposed and control cultures of each of the four test species. In addition, to confirm these results, determinations of cell numbers for cultures of M. aeruginosa, S. capricornutum and N. pelliculosa and of optical density for A. flos-aquae were performed.

Chlorophyll a analyses were conducted according to the procedures of Strickland and Parsons (1972) and involved filtering algal cultures from test medium, extracting chlorophyll by treatment of algal cells with acetone, determining extinction values with a spectrophotometer and finally, calculating the chlorophyll a concentration in the solution. Chlorophyll a and optical density measurements (at 680 nanometers) were made with a Bausch & Lomb Spectronic 20 spectrophotometer. Cell counts were performed with a compound light microscope and a hemacytometer. In each case, the measurements obtained from triplicate exposed cultures were averaged, the results compared with those from triplicated controls and a percentage effect (relative to controls) was calculated.

Each test concentration was converted to its logarithm and the corresponding percentage effect (change in chlorophyll a concentration, optical density or cell number) converted to a probit. The 24-, 48-, and 96-hour median effective concentrations, EC50's (concentrations effective in changing the chlorophyll a concentration, optical density or cell number of exposed algae by 50% as compared to controls) and their 95% confidence limits were then estimated from a linear regression equation calculated with a programmable calculator.

Test methods used for static bioassays with macroinvertebrates and fishes were as described in "Methods for Acute Toxicity Tests

with Fish, Macroinvertebrates, and Amphibians" (U.S. EPA, 1975).

Results of macroinvertebrate bioassays are expressed as EC50's (concentrations effective in causing immobilization of 50% of test animals) and results of fish bioassays are expressed as LC50's (concentrations lethal to 50% of test animals). The EC50 and LC50 values and their 95% confidence limits were estimated from a linear regression equation calculated with a programmable calculator. Data from replicates were averaged and utilized in the regression analysis.

Macroinvertebrate bioassays were conducted in 250-ml beakers containing 200 ml of solution at 20 $\pm$ 1.0°C. Aged well water (hardness, 35 mg/l as $CaCO_3$; pH, 7.1) was utilized in the performance of these bioassays.

Dissolved oxygen values in test vessels during static bioassays with invertebrates ranged from 8.0 to 8.2 mg/l throughout the testing period. Macroinvertebrates were introduced into test beakers within 30 minutes following addition of the RDX; 15 animals of each species were tested at each concentration (3 replicates, 5 animals/ replicate). Static fish bioassays were conducted in 19.6-liter glass vessels held in constant temperature water baths at 20 $\pm$ 1.0°C for the bluegill, channel catfish, and fathead minnow, and at 10 $\pm$ 1.0°C for the rainbow trout. The standard diluent (well water)

for the fish species had a hardness of 35 mg/l as $CaCO_3$ and a pH of 7.1. Dissolved oxygen values in various test vessels during bioassays with fishes ranged from 9.0 initially to 4.0 mg/l at the end of the tests. Fish were introduced into each test vessel within 30 minutes after the compound was added; 30 animals of each species were utilized for each concentration (3 replicates, 10 animals/replicate).

Fathead minnows were chosen as the test species to evaluate the relative susceptibility of life stages of fish to RDX because of the ability to readily procure their various life stages in the laboratory. The susceptibility of selected life stages (egg, 1-hour old newly-hatched fry, 7-day old fry, 30-day old fry, and 60-day old fry) of fathead minnow (Pimephales promelas) to RDX was evaluated under static bioassay conditions for a 144-hour period with the eggs, and for a 96-hour period with all other life stages. The egg, 1-hour old fry and 7-day old fry bioassays were conducted in 250-ml beakers containing 200 ml of solution (10 animals/beaker, 3 replicates/concentration, 30 animals/concentration). The 30-day old fry and 60-day old fry bioassays were conducted in 1-gallon glass jars containing 3 l of solution (10 fry/jar, 3 replicates/ concentration, 30 animals/concentration). The LC50 values for the egg tests were calculated at 24, 48 and 144 hours. The time period of 144 hours allowed 100% hatch of eggs in all control beakers. In addition to percent mortalities, percent hatch of eggs was also observed. These tests were conducted at 25 $\pm$ 1.0°C, and the

standard diluent had a pH of 7.1 and total hardness (EDTA) of 35 mg/l as $CaCO_3$.

Due to their sensitivity to the chemicals, their availability, and their expected presence in most of those areas where RDX might be found, bluegill were selected as the test species to evaluate the effect of water quality on the toxicity of RDX. The susceptibility of bluegill to RDX under various water quality conditions was evaluated during static bioassays for a 96-hour period. The bluegill used in these tests were obtained from a commercial fish farmer in Nebraska, and had a mean ($\pm$ S.D.) wet weight and standard length of 0.9 ($\pm$ 0. , g and 33 ($\pm$ 5) mm, respectively. Bioassays were conducted utilizing bluegill to determine the 24-, 48-, and 96-hour LC50 values of RDX: (a) at three temperatures representing the lower end (15°C), mid-point (20°C), and upper end (25°C) of the normal temperature range for bluegill using soft water (35 mg/l $CaCO_3$) at neutral pH; (b) in soft water water (35 mg/l $CaCO_3$), in hard water (100 mg/l $CaCO_3$) and in very hard water (250 mg/l $CaCO_3$) using water of pH 7.0 at the recommended test temperature of 20°C; and (c) at pH's of 6.0, 7.0 and 8.0 uging standard soft water at the recommended test temperature of 20°C. The diluent for each of these conditions was prepared according to the procedures recommended by Marking and Dawson (1973). Dissolved oxygen values in various test vessels during these bioassays ranged from 9.0 initially to 4.2 at the end of the tests.

General availability and their expected presence in those areas where the RDX might be found resulted in the use of bluegill to evaluate the stability of the toxicological properties of RDX. The susceptibility (LC50) of bluegill (Lepomis macrochirus) to RDX was evaluated under static bioassay conditions for a 96-hour period utilizing solutions which were "aged" for 0, 12, 24, 48, and 96 hours. Fish (10 fish/replicate, 3 replicates/concentration, 30 fish/concentration) were introduced into aged test solutions at each time period. The bluegill used in these tests were acquired from a commercial fish farmer in Nebraska, and had a mean (± S.D.) wet weight of 0.8 (± 0.2) g and a mean (± S.D.) standard length of 32 (± 4) mm. The standard diluent had a pH of 7.1 and a total hardness (EDTA) of 35 mg/l as $CaCO_3$.

(B) Flow-through Acute Toxicity Tests - Procedures used in these toxicity tests were based on protocols described in "Methods for Acute toxicity Tests with Fish, Macroinvertebrates, and Amphibians" (EPA, 1975) except where stated otherwise.

The flow-through bioassays were conducted using an intermittent-flow proportional dilution apparatus (Mount and Brungs, 1967). The apparatus provides for intermittent introduction of seven (fish) and six (invertebrates) concentrations of the test compound into 30-liter (fish) or 1.75 l (invertebrates) test vessels and diluent water to a vessel serving as a control unit. The control

vessel received solvent (DMSO) at a concentration equivalent to the greatest amount of DMSO introduced to any test vessel. During bioassays with fishes, the flow rate of test solutions to each 30-l aquarium was 5 l/hour throughout the test period. During the flow-through invertebrate bioassays, flow rate to each 1.75 l aquarium was 4.0 l/day. Thirty fish were randomly assigned to each test vessel within 30 minutes after the test was initiated. Twenty water flea or midge larvae were randomly assigned to each replicate test chamber within 30 minutes after the test was initiated.

Diluent water used in these tests had the same water quality characteristics as previously described for holding water. All test vessels were maintained in water baths at 21 $\pm$ 1.0^{o}C and test solutions were not aerated during the test. During these tests, the dissolved oxygen concentration, pH and temperature of test solutions were checked at various intervals during exposure in the highest, middle and lowest test concentrations, at a minimum. DO and temperature were measured with a YSI dissolved oxygen meter and combination oxygen-temperature probe; pH was measured with a Corning Digital pH meter and probe.

Results of the tests were expressed as the time-dependent (24- and 96-hour) LC50's, and time-independent (incipient)

LC50, the nominal concentration of test compound in diluent water which caused 50% mortality in test populations of fish and invertebrates with no additional significant response (<10%) during the final 48 hours of exposure. Test concentrations and corresponding observed percentage mortalities were converted to logarithms and probits, respectively, and these values were utilized in a least squares regression analysis. The LC50's and their 95% confidence intervals were calculated from the regression equation.

(C) Bioaccumulation Of RDX In Fishes - A modified intermittent-flow, dilution apparatus (Mount and Brungs, 1967) was used to deliver a nominal concentration of 0.01 and 1.0 mg/l 14C-RDX in Bionomics' well water, and control water (well water) to three, 30-liter glass aquaria at a rate of four aquarium volume exchanges/ day.

The 14C-RDX stock solutions to be used in these studies were prepared by dissolving the entire contents of the vial containing the 14C-RDX in 100 ml of DMSO. The 0.01 mg/l 14C-RDX exposure level super stock was subsequently formulated by transferring 20 ml of the above solution (400 uCi and 26.14 mg RDX) to a 100 ml volumetric flask, adding 2.27 ml of the unlabelled RDX stock (78-86 mg RDX), and diluting to volume with DMSO, yielding a 14C-RDX concentration of 1.05 mg/ml. The 1.0 mg/l 14C-RDX super stock was prepared by transferring 20 ml of the above solution (400 uCi and

26.14 mg RDX) to a 100 ml volumetric flask, adding 30.10 ml of the unlabeled RDX stock (10,473.9 mg RDX), and diluting to volume with DMSO, yielding a concentration of 105 mg/ml.

To determine the specific activity of the 14C-RDX in each super stock, three 23-microliter (ul) aliquots of each super stock were transferred to glass scintillation vials containing 15 ml of Monophase (a xylene base counting solution with nonionic surfactants and PPO + bis/MSB scintillators, Packard Instrument Co.), placed in a Model 2112 Packard Tri-Carb Liquid Scintillation Spectrometer and the activity measured. The mean specific activities of the 0.01 mg/l and 1.0 mg/l 14C-RDX super stocks were determined to be 11,029 $\pm$ 208 and 112 $\pm$ 1 disintegrations per minute/microgram (dpm/ug), respectively, equivalent to 132 $\pm$ 2% of the theoretical specific activity of each super stock.

Diluter stock solutions were periodically prepared for both 14C-RDX exposure levels by dissolving 15.2 ml of the appropriate super stock in four liters of DMSO. A container to promote mixing of the 14C-RDX from the diluter stock and the dilution water was utilized prior to delivery of the test solution to the exposure tanks.

On June 15, 1976, 65 bluegill and channel catfish and 130 fathead minnow were placed in each aquaria to initiate the exposure period. Fish in all aquaria were fed Agway Strike dry pelleted ration ad libitum daily and fecal material was siphoned every other day. The water

temperature in each aquarium was maintained at 18 ± 1°C. In order to maintain a dissolved oxygen concentration >60% of saturation, the test water was continuously aerated.

Water and fish samples were taken from the exposure aquaria on days 0,1,3,7,10,14,21, and 28 of fish exposure. Control water samples were taken on days 3 and 28 of exposure. On each sampling day, triplicate 5.0 ml water samples were pipetted directly into scintillation vials containing 15 ml of counting solution. The vials were then placed in the Liquid Scintillation Spectrometer for quantitation of 14C-RDX.

Three bluegill, three catfish and six fathead minnows were removed from both 14C-RDX exposure aquaria on days 1,3,7,10,14,21 and 28, of the exposure period and on days 1,3,7,10 and 14 of the depuration period. Control fish were removed on days 3, and 28 of exposure. Each bluegill and catfish sampled was eviscerated and filleted while the minnows were eviscerated and beheaded but not filleted, for determination of 14C-residues in muscle (fillet, or beheaded and eviscerated fish) and visceral tissues. Duplicate samples of the edible tissue of each species and the viscera in its entirety from each bluegill and catfish, and the combined viscera from the minnows were wet weighed and air-dried for *ca* 24 hours in combustion cones at 21°C. Each dried sample was then combusted in a Packard Model 306 Sample Oxidizer. The resulting $14CO_2$ was trapped as a carbonate in a mixture of Carbosorb (1M hyamine hydroxide in methanol)

and scintillation fluid (4 g 98% PPO + 2% bis/MSB/liter toluene) into a scintillation vial. The vials were then placed in the spectrometer and the activity determined.

Recovery rates of the oxidizer were determined prior to analyzing each set of samples by combusting and counting the activity of a standard reference material (New England Nuclear Corp.) and comparing the measured value to the known theoretical value of the standard. Recovery rates were 99-101% and experimental data were not adjusted for percentage recovery. The counting efficiency (E) for each sample was determined as follows. Aliquots of a 14C-toluene standard, obtained from New England Nuclear, with an accurately known specific activity were added to a series of counting vials containing counting solution of a type and volume identical to those used for counting water or combusted tissue samples. Increasing volumes of nitromethane, a chemical quenching agent, were then added to individual vials in each series. The vials were counted with two channels simultaneously and the channel ratios determined. The counting efficiency of each standard was determined as the quotient of counts per minute divided by the disintegrations per minute of the standard. From these data, a graph of counting efficiency versus channel ratio was constructed for each of the two types of samples (water and tissue). Counting efficiencies of all test samples were determined by calculating the channel ratio and interpolating the corresponding counting efficiency from the graph

(Rapkin, 1970). By this method, counting efficiencies for 14C-RDX ranged from 0.77-0.82 for water and from 0.51-0.77 for fish tissue.

Background levels of radiation for water and fish were determined by analyzing control samples prior to the initiation of the study and were measured to be 39 dpm for water and 36 dpm for fish tissue. All samples were counted for a minimum of 100 minutes or until 5,000 counts were generated. Using this procedure and the calculations described in Standard Methods for the Examination of Water and Wastewater (APHA, 1971) it was determined (at the 95% confidence level) that the minimum detectable activity above mean background levels (minimum net cpm) was 22 cpm. A 7.9% counting error is associated with this value. This is the maximum counting error, as the percentage counting error for each sample depends on the net cpm of that sample and varies inversely with the activity. The minimum detectable 14C-residue concentration for edible tissue is a range as it depends on the efficiency and weight of each sample. The end points of the range are calculated by using the lowest efficiency and lightest sample weight measured (maximum limit) and the highest efficiency and the heaviest sample weight (minimum limit). The detection limits for the tissue samples from the 0.01 mg/l 14C-RDX exposure level were: bluegill viscera 0.001-0.006 mg/kg; bluegill muscle 0.0007-0.004 mg/kg; catfish viscera 0.001-0.006 mg/kg; catfish muscle 0.001-0.004 mg/kg; minnow viscera 0.001-0.008 mg/kg; and minnow muscle 0.001-0.005 mg/kg.

The detection limits at the 1.0 mg/l exposure level were: bluegill viscera 0.70-0.76 mg/kg; bluegill muscle 0.08-0.27 mg/kg; catfish viscera 0.14-0.70 mg/kg; catfish muscle 0.13-0.61 mg/kg; minnow viscera 0.26-1.2 mg/kg; and minnow muscle 0.13-0.45 mg/kg.

The calculations used in determining the concentration of 14C-RDX or 14C-residues in each sample were the following:

i. net counts/minute (cpm) = (sample-background) cpm

ii. counting efficiency (E) = from channel ratio method

iii. disintegrations per minute (dpm) in counted sample = $\frac{\text{net cpm}}{\text{counting efficiency (E)}}$

iv. total 14C-residues calculated as RDX (ug/l) (ug/ml or ug/l) = $\frac{\text{dpm in combusted sample}}{\text{specified activity of the RDX superstock x sample size (dpm/ug) x (g or ml)}}$

Sample Calculation

$\frac{\text{net cpm (619)/E (0.57)}}{\text{specific activity x sample wt.}}$ = 2.3 mg/kg 14C- residues calculated as RDX

(746) (0.616 g)

Each set of daily sample measurements was examined for "outliers", or extreme observations, according to the method of Dixon and Massey (1957). Any values determined not to be from the same

"population" as the other values in that set (P=0.05) were discarded. A mean 14C-RDX concentration was then calculated from the measured activity of the samples determined for each sampling day.

(D) Critical Life Stage Studies - The effects of 30 days continuous exposure to RDX on the eggs and fry of channel catfish and fathead minnows were investigated using the procedures recommended by U.S. EPA (1972). A proportional diluter (Mount and Brungs, 1967) modified with a McAllister (1972) chemical metering device delivered water and toxicant at a dilution factor of 0.5 to the test aquaria. Chemical stock solutions were dissolved in DMSO to a volume of two liters. Stock solutions were prepared three times during the thirty day exposure. Five concentrations, a control and solvent control flowed through separate glass delivery tubes to duplicate test aquaria. Thus, each concentration and each control was replicated and each replicate was designated as either A replicate or B replicate. Test aquaria measured 30 x 35 x 30 cm. Each test aquarium contained two growth chambers (13 x 30 x 27 cm) designated A and B, which had stainless steel 40 mesh screen affixed to one end allowing water to drain out while retaining the young fry. A constant-level drain tube extending 15 cm above the bottom of each test aquarium retained a total volume of 15.75 l. Each growth chamber contained a water volume of 5.9 l. A glass, flow-splitting chamber was calibrated to deliver an equal volume to each of the growth chambers. The test water was delivered to all chambers at

a mean flow of 6 tank volumes for each species per 24 hours.

A constant temperature of 22 $\pm$ 1^{o}C was maintained for test organisms by placing aquaria in water baths in which circulating water was heated by immersion coil heaters and regulated by mercury column thermoregulators.

Exposure of channel catfish eggs began within 48 hours after fertilization. Eggs were obtained from a catfish farmer in Missouri. Fifty eggs were randomly distributed to each egg cup. In order to retard fungus, eggs were treated each day during the first three days of exposure with a 3 minute dip in a 30 ppm solution of malachite green. Dead eggs were removed and counted each day until hatching was completed (7-8 days after exposure began at 25^{o}C). Subsequently, 25 fry were randomly selected and transferred to the larval growth chambers. Fry were fed brine shrimp nauplii ad libitum twice per day and Agway Strike Trout Starter once per day, beginning 5 days after hatching was completed and continuing throughout the larval exposure period.

Exposure of fathead minnow eggs began within 48 hours after fertilization. Eggs were obtained from brood stocks at the Aquatic Toxicology Laboratory of E G & G, Bionomics in Wareham, Massachusetts.

Forty eggs were randomly distributed to each egg cup. Dead eggs were removed and counted each day until hatching was completed (3-5 days after exposure began at 22°C). After hatching was completed, and based on the percent hatch, fry were randomly selected and transferred to the larval growth chambers. Fry were fed brine shrimp nauplii ad libitum twice per day, beginning one day after hatching was completed and continuing throughout the exposure period.

For both species the two egg cups were oscillated in their respective test vessels by means of an egg cup rocker arm apparatus (Mount, 1968). Percentage hatch was based on the number of live fry in the egg cup when hatching was completed. After fry were transferred, growth chambers were siphoned twice weekly to remove fecal aterial and excess food. For each group of fry, total length as determined at 30 days post-hatch using the photographic method of McKim and Benoit (1971). Percentage survival at 30 days post-hatch was also recorded at this time.

Based on acute toxicity information, nominal concentrations were established for both tests. Catfish were exposed to nominal RDX concentrations of 2.0, 1.0, 0.50, 0.25 and 0.12 mg/l. Fathead minnows were exposed to nominal concentrations of 5.0, 2.5, 1.3, 0.63 and 0.31 mg/l of RDX.

At the initiation of each test, the highest concentration of DMSO in the test chambers was 420 mg/l, which is 0.013 of the

LC50's reported by Wilford (1967) for several species of freshwater fish.

Temperature and dissolved oxygen were monitored daily at all concentrations and controls using a YSI dissolved oxygen meter and probe. The pH was measured weekly in the high and low treatment levels and controls using a Corning Digital 109 pH meter. Each duplicate aquaria was sampled on a rotating basis, enabling alternating measurement of all aquaria. Total hardness was measured once at the high and low treatment levels and controls by the EDTA titrimetric method (APHA, 1971). Samples were taken for analysis of RDX from the same replicate in the highest, middle, and lowest concentration once each week. All samples were taken at a point from each fry chamber approximately midway between the surface, bottom, and sides and composited into one sample for each duplicate.

The means of the measured biological parameters (from duplicate aquaria) were subjected to analysis of variance according to Steel and Torrie (1960). The data for percentage survival and percentage hatch were transformed to arcsin$\sqrt{\text{percentage}}$ prior to hatch. When treatment effects were indicated, the means of these effects were subjected to Dunnett's procedure for comparing treatment means with control (Steel and Torrie, 1960). All differences were considered statistically significant at a probability of P=0.05.

(E) Invertebrate Chronic Tests - To investigate the chronic toxicity of nitroglycerine to both midge larvae and daphnids a proportional

dilutor (Mount and Brungs, 1967) with a dilution factor of 0.5 and a syringe injector, delivered the test water and test compound to the mixing chamber, and mixing cells. From the mixing cells, the RDX solutions were delivered to each of 4 replicate aquaria through individual glass delivery tubes. RDX stocks were prepared in DMSO. Two of the 4 control replicate aquaria received doses of DMSO identical to the greatest amount of DMSO delivered to the RDX treated vessels, to detect any possible DMSO related effects. The concentration of DMSO in these aquaria was 300 mg/l.

Each experimental unit consisted of cylindrical glass battery jars 18 cm high and 13.5 cm wide. A 3 x 8 cm notch was cut into the top edge of the aquaria and covered with Nytex^{R} 40 mesh screen to provide drainage. Cylindrical cages constructed of aluminum 16 mesh screen were affixed to the battery jars to allow for adequate emergence areas for the adult midges.

The water depth within the aquaria was 15 cm and the volume was 1.75 l. Fifty ml of the solution was delivered to the individual test vessels every 25 minutes during the water flea exposure and every 8 minutes during the midge larvae exposure. For both species, the initial 2 days of the exposures were maintained under static conditions to prevent damage of the early instar organisms due to turbulence.

Dissolved oxygen and temperature were measured bi-weekly throughout these exposures with a YSI dissolved oxygen meter equipped with a combination oxygen-temperature probe. Water samples were taken for analysis of the RDX concentrations from 2 replicates of all concentrations weekly, on a rotating basis. This enabled alterating measurement of all replicate concentrations. Daphnia magna (≤24 hours old), procured from laboratory stock cultures, were used to initiate the chronic exposure. Twenty water flea were randomly assigned to each test aquarium. Observations of survival and production of young were made weekly for each test aquarium. If young were present, they were counted and discarded. On day 21, twenty young from each aquarium were retained for the initiation of the second generation exposure.

Chironomus tentans (≤48 hours old), procured from laboratory cultures were used to initiate this chronic exposure. One hundred organisms were placed in each test vessel. Prior to the introduction of the test organisms, the aquaria were supplied with a substrate consisting of homogenized paper towel, in water, approximately 1 cm deep, and one hundred 2 and 3 mm diameter sections of glass tubing, 2 to 3 cm long. Early instar midge larvae utilize the paper towel substrate for construction of dwelling tubes, while late instar larvae, generally, utilize the glass tubing, where their numbers can be visibly quantitated.

Determination of the survival of larvae was made after the control animals had entered their 4th instar stage. Beginning with the

onset of emergence, daily records were kept of emergence, adult survival, pupae survival, and egg production. The data were cimpiled up to the day at which adult mortality of the control animals was greater than emergence of these controls. At this point the aquaria were cleaned, new substrate was supplied, and the second generation was initiated with larvae originating from egg masses taken from the same treatment level into which they were placed.

The food supplied in these toxicity tests consisted of homogenized Agway Strike trout starter food and cerophyll^{R} (20:1 ratio). The combination was blended in water and was filtered through a stainless steel 102 mesh screen for removal of large particles prior to use. Aliquots ranging from 0.2 to 0.4 ml of this solution, depending upon the clarity of the water, were pipetted into each aquarium three times daily.

Means of the measured biological parameters from duplicate aquaria were subjected to analysis of variance (completely randomized block design P=0.05). The data for percentage survival and percentage hatch were transformed to arcsin$\sqrt{\text{percentage}}$ prior to analysis. When treatment effects were indicated these means were subjected to Dunnett's procedure (Steel and Torrie, 1960). When a treatment mean was significantly different (P=0.05) from the control mean, that treatment level was considered a toxic effect level.

(F) Fathead Minnow Chronic Study - To investigate the chronic toxicity of RDX to fathead minnows we closely followed the

recommended bioassay procedures for fathead minnow chronic tests issued by the Environmental Research Laboratory, Duluth, Minnesota (U.S. EPA, 1971b).

A proportional diluter (Mount and Brungs, 1967) with a dilution factor of 0.5 was used to deliver five concentrations of RDX and two controls to duplicate test aquaria. One control received only the diluent water (negative control) and a second control received a volume of DMSO (positive or solvent control) equal to that which was added to the aquaria containing the highest concentration of RDX and DMSO to which fish were exposed. A stock solution of RDX, dissolved in DMSO, was delivered to the mixing container from a 50 ml gas-tight syringe with a stainless steel needle. The amount of DMSO added to the highest concentration of RDX and to the solvent control was 26 mg/l. This is approximately 0.001 of the LC50's reported by Wilford (1967) for DMSO and several species of freshwater fish.

A flow-splitting chamber (Benoit and Puglisi, 1973) was used to promote mixing of the RDX and diluent water prior to delivering test water to the aquaria through glass tubing. Each glass aquarium (90 x 30 x 30 cm) was subdivided by a stainless steel 40 mesh screen to provide space for two fry chambers (30 x 12 x 25 cm) and a spawning chamber. Each aquarium was duplicated, resulting in two aquaria, four fry chambers and two spawning chambers for each test concentration. The water level in each

aquarium was maintained at 15 cm by a standpipe. The flow rate to the duplicate spawning and quadruplicate fry chambers was eight times their volume per 24 hours.

Five spawning tiles made from halved, 7.5 cm-wide sections of 10 cm diameter cement-asbestos drain tile were placed in each spawning chamber with the concave surface downward. Egg groups were incubated in "egg cups" made from 5 cm diameter glass jars with Nytex^{R} 40 mesh screen bottoms. The egg cups were oscillated in the test water by means of a rocker-arm apparatus driven by a 2 rpm motor (Mount, 1968).

A constant temperature of 25 $\pm$ 1^{o}C was maintained in the aquaria by placing them in water baths within which ciruculating water was heated by immersion coils and regulated by a mercury column thermoregulator.

The photoperiod followed the normal daylight hours of Evansville, Indiana (U.S. EPA, 1971b) and was adjusted on the first and fifteenth day of each month beginning with the Evansville daylength of December 1st on the first day of the test as suggested in the protocol. Illumination was provided by a combination of Durotest (Optima F.S.) and wide spectrum Grow Lux fluorescent lights located centrally, 64 cm above the surface of the water in the aquaria. The entire experimental unit was screened with black, polyethylene curtains to prevent disturbance of the fish and to minimize the effect of ambient laboratory lighting on the intended photoperiod.

Temperature and dissolved oxygen concentrations were measured daily, using a YSI dissolved oxygen meter with a combination electrode-polarographic probe, in test aquaria on a rotating basis so that all aquaria were checked at least once each week. Total hardness, alkalinity, pH and acidity were measured in each concentration during the test. Initially, each duplicate tank from the five exposure concentrations of RDX was sampled weekly. After eight weeks, each duplicate was sampled every other week, and after 15 weeks, samples were taken monthly until the test was ended.

The initial chronic exposure of fathead minnows to RDX began on October 11, 1975 with eggs obtained from the Environmental Research Laboratory in Duluth, Minnesota. This test ended on March 30, 1976 after all fish were accidentally killed by a treatment for external parasites administered on test day 140. A subsequent chronic exposure was started on June 15, 1976 with eggs obtained from Duluth, Minnesota. In both chronic tests the following biological procedures were utilized.

One group of sixty eggs was exposed in each duplicate aquarium and dead eggs were counted and removed daily until hatching was completed. Percentage hatch (number of live fry/60 eggs x 100) was calculated for each duplicate. Two groups of twenty fry from each egg group were randomly selected and placed in the respective fry chambers.

During the initial 30 days of exposure fry were fed live brine shrimp (Artemia salina) nauplii four times daily. During the 30 to 60 days exposure interval, minnows were fed three times daily with frozen brine shrimp. After 30 and 60 days of post-hatch exposure, fry groups were transferred onto a transluscent millimeter grid and photographed for determination of total lengths. Percentage survival was also recorded at these intervals. On day 64 (60 days post-hatch), fish from the two fry groups in each aquarium were combined and 15 fish were impartially selected to continue exposure in each duplicate spawning chamber. The remaining fish from each duplicate were frozen for possible tissue analysis. While in the spawning chambers, fish were fed twice daily with frozen brine shrimp supplemented with live Daphnia and Agway Strike trout food granules. All tanks were siphoned weekly to remove particles and brushed at intervals of 8-10 weeks when algal growth became excessive.

On test day 181 most fish had developed well defined secondary sexual characteristics and fish in each aquarium were sexed and their number reduced to three males and seven females. A few tanks contained fish which began spawning before the thinning was done, but spawning began in most tanks during the week which followed day 181. Eggs were removed from the underside of spawning tiles after 1:00 P.M. each day by applying gentle pressure and a rolling motion of the finger tips to the adhesive egg mass. Eggs

from each spawn were counted and groups of fifty eggs from the first seven spawns in each aquarium were incubated for hatchability determinations. Once hatchability was determined for seven spawns in each aquarium only eggs from every third spawn were used for hatchability determinations and no weekend spawns were incubated.

Twenty fry from the first two egg groups successfully incubated in each aquarium were placed in the respective fry chambers and reared for 30 days as described previously for first generation fish. At the end of 30 days each group was photographed for determination of total length, and wet weighed.

Exposure of adult fish was terminated on day 240 when spawning activity had decreased considerably and egg production indicated that exposure concentrations had not adversely affected spawning in any aquaria. Each adult fish was individually measured, wet weighed and examined for sex and degree of sexual maturity.

Means of measured biological parameters from duplicate aquaria were subjected to analysis of variance (completely randomized block design, P=0.05). Data for percentage survival and percentage hatch were transformed to arcsin$\sqrt{\text{percentage}}$ prior to analysis. When treatment effects were indicated, the means of these parameters were compared to control using Dunnett's procedure (Steel and Torrie, 1960).

Chemical Methods

Water samples were collected and 200 ml portions, measured volumetrically, were stored in amber glass bottles with teflon-lined screw caps prior to analysis by gas/liquid chromatography (GLC). The water sample was added to a 500 ml separatory funnel, equipped with a teflon stopcock, and 80 ml of nanograde quality benzene was added to the amber glass storage bottle. The bottle was capped and shaken to extract into the benzene solvent any RDX which may have absorbed to the glass surface. The benzene was then added to the water sample in the separatory funnel and shaken vigorously for one minute. Emulsification was not observed and phase separation was complete within 10 minutes.

The water was drained from the separatory funnel and the benzene was added to a glass-stoppered storage bottle. Benzene extracts of water containing in excess of 1 mg RDX per liter were analyzed by GLC. Extracts of other samples containing less than 1 mg RDX per liter were concentrated to less than 25 ml, using high vacuum rotary evaporation (at room temperature), the extract was diluted to 25 ml with benzene, and finally an aliquot was analyzed by GLC.

The GLC operating conditions were as follows:

Instrument: Perkin-Elmer Model 3920 equipped with a 6' x 2 mm I.D. glass column coated with 3% Dexsil 300 GC on 100/120 mesh Supelcoport.

Detectors: Electron capture (ECP) using 15 mCi ^{63}Ni and rubidium thermionic (specific for nitrogen and phosphorus).

Temperatures (^{o}C)

Inlet:	220	ECD:	325
Column:	185	Thermionic detector:	260
Outlet:	260		

Gas flows

Column: 35cc N_2/min

Splitter: 1 part to ECD; 17 parts to thermionic

Make-up gas to ECD: 50cc N_2/minute

Reactant gas to thermionic: 8cc H_2 and 100cc air/minute

Recorder: Leeds & Northup Model 620 dual pen, operated at 0.5 cm/minute chart speed and 0-1 mV sensitivity.

Response: 8 nanograms of RDX gave 32 and 4 percent of full-scale response with ECD (attenuation X2) and thermionic (attenuation X1), respectively. RDX eluted from both detector systems in 3.4 minutes, measured from the benzene solvent front.

Five quality control samples containing 1.5 mg RDX/l were manufactured by adding 300 micrograms of RDX in 1 ml of acetone to 200 ml portions of well water from E G & G, Bionomics. An aliquot of the benzene extract was analyzed without prior solvent concentration and the average recovery was 68.0 $\pm$ 13 percent. Five additional

quality control samples containing 0.25 mg RDX/l were manufactured by adding 50 micrograms of RDX in 1 ml of acetone to the water sample, extracting the samples and concentrating the benzene extract from 80 ml to 25 ml using high vacuum rotary evaporation. The average recovery by this method was 63.0 $\pm$ 3.5 percent. These method recovery percentages were applied to the appropriate raw data (according to the method utilized) to calculate actual concentrations.

RESULTS

(A) Static Acute Toxicity Tests - RDX was generally less toxic to invertebrates and algae than to fishes during static acute toxicity tests (Tables 2,3 and 4). All of the invertebrate species exhibited EC50's greater than (>) 100 mg/l, and all of the algae species exhibited EC50's >32 mg/l. The chlorophyte Selenastrum capricornutum appeared to be the most sensitive algae tested. RDX was considerably more toxic to fishes (range of LC50's, 4.1-6.4 mg/l) with little difference in susceptibility exhibited between species.

The acute toxicity of RDX to various life stages of the fathead minnow varied considerably (Table 5). The egg stage was unaffected by concentrations as high as 56 mg/l. The 1-hour old fry were slightly more susceptible to the 96-hour exposure while the 7-day old fry were the most susceptible life stage tested (96-hour LC50, 3.8 mg/l). The 30- and 60-day old fry exhibited similar susceptibilities through the 96-hour exposure.

The acute toxicity of RDX to bluegill was essentially unaffected by variations in water quality (Table 6). The toxicity appeared to be slightly less at the lowest temperature tested.

Aging solutions of RDX for periods ranging from 24 to 96 hours had no effect on the toxicity to bluegill (Table 7). The calculated LC50 values were essentially identical in every instance, indicating

that concentrations of RDX are stable throughout the duration of the 96-hour standard toxicity test, at a minimum.

(B) Chemical Analysis Of RDX In Water - The result of gas chromatographic analysis of water samples to quantitate RDX concentrations from all experiments indicate that the mean measured concentrations very closely approximate the nominal concentrations. In addition, these analyses show a high degree of stability of RDX in water. Radiometric analyses of water samples from the bioconcentration study showed that there is little variance between nominal and measured concentrations of RDX.

(C) Flow-through Acute Toxicity Tests - Dissolved oxygen concentrations during flow-through acute toxicity tests with both fishes and aquatic invertebrates were >60% of saturation. RDX exhibited no toxic action on aquatic invertebrates during 96 hours of exposure at a high concentration of 15 mg/l (Table 8). With the exception of channel catfish, the fishes exhibited essentially the same response to continuous exposure to RDX as was observed during static acute toxicity tests. The LC50 for catfish after 264 hours of exposure was ca. X greater than that observed after 96 hours of exposure in static acute toxicity tests.

(D) Bioaccumulation Of RDX In Fishes - Throughout the exposure and depuration periods, fish in all tanks appeared normal and fed readily, but the percentage mortalities among bluegill and minnows at both exposure levels was high. Twenty six percent of the bluegills introduced were killed at both 14C-RDX exposure levels while 23 and 22% of the minnows were killed at the 0.01 and 1.0 mg/l 14C-RDX exposure levels, respectively. The percentage mortality among bluegill and minnow control fish was 2% for both species. The percentage mortalities of catfish were 3 and 2% for fish exposed to 0.01 and 1.0 mg/l 14C-RDX respectively and 14% for control fish. Post-mortem examinations of all species revealed no parasite infestations or any tissue abnormalities, but as a precautionary measure, all fish were treated (one application) with 25 ppm of formalin and 0.1 ppm malachite green on five consecutive days beginning July 1. This treatment did not appear to reduce mortalities.

The mean measured 14C-residue concentrations in the water of the 0.01 mg/l exposure aquarium are presented in Table 9. Diluter malfunctions prior to sampling on days 7 and 10 greatly increased the measured concentrations on these days and increased the mean measured 14C-RDX exposure concentration to 0.014 $\pm$ 0.008 mg/l, 40% above nominal.

The mean measured 14C-residue concentrations (calculated as RDX) in the muscle and visceral tissue of bluegill, catfish and minnow

exposed to a 14C-RDX concentration of 0.014 mg/l for 28 days and during 14 days depuration are presented in Tables 10, 11, 12, and Figure 1. The test for "outliers" or extreme observations (Dixon and Massey, 1957) revealed no extraneous values for any set of daily sample measurements.

From the data it is evident that accumulation and elimination of 14C-residues was similar among all three species and was directly proportional, although slightly delayed, to the concentration of 14C-residues in the water. To account for the variation of 14C-residue concentrations in the water, a bioconcentration factor (concentration of 14C-residues in tissue divided by the concentration in the water) was calculated for both the muscle and visceral tissue of each species on each sample day (Tables 10, 11, 12 and Figure 2). These data more appropriately describe the true rates of accumulation and elimination of 14C-residues. Based on these data it appears that steady-state, or equilibrium between the rates of accumulation and elimination, in the muscle of each species over a period of time was reached by day 14 and maintained until termination (day 28). Based on the mean of the bioconcentration factors calculated during this period of equilibrium, the equilibrium bioconcentration factors for 14C-RDX in the muscle of fish exposed to a concentration of 0.014 mg/l 14C-RDX are estimated to be ca 5X for bluegill and minnow and 4X for catfish.

Accumulation of 14C-residues in the viscera followed a similar trend, reaching equilibrium on day 14 (Tables 10, 11 and 12).

The equilibrium bioconcentrations for 14C-RDX in visceral tissue are estimated to ba ca 10X in bluegill, 11X in minnow, and 6X in catfish.

The mean measured 14C-residue concentration in the water of the 1.0 mg/l exposure aquarium remained virtually constant throughout the exposure period with the exception of day 14 (Table 9). The large decrease on that day was due to a diluter malfunction and was only temporary.

The mean measured 14C-residue concentrations (calculated as RDX) in the muscle and visceral tissues of bluegill, catfish and minnow exposed to a 14C-RDX concentration of 1.0 mg/l for 28 days and during 14 days depuration are presented in Tables 10, 11, 12 and Figure 3. To account for the substantial decrease of 14C-residues in the water on day 14 bioconcentration factors in muscle were calculated and graphed with time (Figure 4). From these data it is apparent that a steady-state had been reached in the bluegill after only one day of exposure while equilibrium was not attained in minnow muscle until 14 days of exposure. It appears that equilibrium was also reached in the catfish muscle after 21 days of exposure, but additional data from continued exposure would be needed to substantiate this occurrence. Based on the bioconcentration factors calculated during the period of equilibrium in bluegill and minnows, the equilibrium bioconcentration factor for

14C-RDX is estimated to be ca 4X. The maximum bioconcentration factor for 14C-RDX in catfish muscle is estimated to be 3X.

The bioconcentration factors calculated for 14C-RDX in the viscera of bluegill and catfish exposed to a nominal 14C-RDX concentration of 1.0 mg/l were fairly constant throughout the exposure period (Tables 10 and 11). Consequently, mean bioconcentration factors utilizing all data points were calculated and determined to be ca 9X for bluegill and 3X for catfish. The bioconcentration factors for 14C-RDX and minnow viscera increased substantially during the last seven days of exposure, yielding a maximum bioconcentration factor of 9X.

After 14 days depuration in flowing, uncontaminated water, virtually all of the accumulated 14C-residues had been eliminated from the muscle and visceral tissue of bluegill and catfish which had been exposed to a 14C-RDX concentration of 0.014 mg/l. During this same period, only 50 and 71% of the accumulated 14C-residues in the muscle and visceral tissue, respectively of the minnows had been eliminated.

Elimination of 14C-residues from fish exposed to a nominal 14C-RDX concentration of 1.0 mg/l was much less extensive. The catfish and minnows had eliminated 82 and 70%, respectively of the 14C-residues accumulated on the muscle and 87 and 84% of the 14C-residues in the viscera, after 14 days depuration. Bluegill did not

appear to eliminate any 14C-residues from either the muscle or viscera during the 14-day depuration period.

(E) Toxicity To Critical Life Stages Studies - During the critical life stage studies with both channel catfish and fathead minnows, the water hardness ranged from 28-32 mg/l as $CaCO_3$, and dissolved oxygen ranged from 49-100% of saturation. Based on the mean measured concentration of RDX in water in the high, middle and low exposure levels (Table 13), the concentrations in the intermediate levels were extrapolated. The percentage hatch of channel catfish eggs was unaffected by measured concentrations of RDX as high as 2.3 mg/l (Table 14). Exposure for 30 days of channel catfish fry to 2.3 and 1.2 mg/l of RDX significantly reduced survival. However, virtually all of the mortality of fry in these concentrations occurred within a 6-day period after a diluter malfunction (10 days post hatch) which may have significantly increased the concentration of RDX in water. Total lengths of 30 day old fry were similar for controls and all concentrations of RDX to which fry were exposed.

No significant differences in percentage hatchability of fathead minnow eggs were observed among egg groups incubated in the control, solvent control, or measured concentrations of RDX as high as 5.8 mg/l. The mean total length of 30 day old fry continuously exposed to 5.8 mg/l was significantly lower than mean total length in control and solvent control. (Table 15).

(F) Chronic Toxicity To Invertebrates - During the chronic studies with water flea and midge larvae, the dissolved oxygen concentrations

ranged from 77% to 85%, and from 56% to 65% of saturation, respectively. The mean measured concentrations of RDX in water are presented in Table 16.

The chronic exposure of D. magna to concentrations of RDX ranging from 1.4 to 20 mg/l had no effect on the survival of these organisms throughout the first generation exposure period (Table 17). The average number of young produced per parthenogenetic female water flea between days 7 and 14 was significantly (P=0.05) reduced at 4.8, 9.5 and 20 mg/l RDX measured concentrations (Table 18). From day 14 to termination of this generation's exposure (day 21) young daphnid production was comparable to the controls in all RDX treated vessels.

Survival and the average number of young daphnids produced per parthenogenetic female during the second generation exposure was comparable to the controls at all RDX treatment levels through the duration of this 3 week exposure (Tables 17 & 18). No significant differences in survival and reproduction were observed between the negative control vessels and the positive (DMSO treated) control vessel during this chronic exposure and were therefore combined.

The chronic exposure of C. tentans to concentrations of RDX ranging from 1.3 to 21 mg/l measured concentrations, for one generation, had no statistically significant RDX-related effects on larvae, pupae, adult survival or adult emergence (Table 19).

On day 14, complete mortality of larvae in the C replicate of the RDX treatment level of 10 mg/l occurred. At this time it was observed that solid particles of RDX had precipitated in the mixing chamber of the dilution apparatus and had been delivered to this test vessel. It appears that the actual concentration of RDX in this vessel was considerably higher than the measured concentration and was responsible for the death of these organisms.

The average number of eggs produced per adult C. tentans per treatment concentration during this first generation exposure is presented in Table 20. Because no appreciable egg production had occurred at many treatment levels after the alloted 23 days of this exposure, egg collection was continued to day 27. No fertile eggs were received from 1.3 and 4.0 mg/l RDX and no eggs were obtained from adults exposed to 10 mg/l RDX. The second generation exposure of these treatment levels were initiated with control eggs.

During the second generation exposure of C. tentans to RDX, no significant survival differences from the controls were observed with larvae, pupae, and adults at 2.2, 10 and 21 mg/l RDX (Table 21). Percent emergence of adults exposed to 2.2 mg/l RDX was significantly lower than the controls; however, we do not attribute this to the presence of RDX because emergence of adults in RDX treated vessels containing greater amounts of this compound were comparable to emergence in control vessels.

Larvae survival in all RDX concentrations was significantly less than the controls. Adult survival was also significantly less at 1.3 and 4.0 mg/l RDX. It is difficult to relate these observations to the presence of RDX since 1.3, 4.0 and 10.0 mg/l RDX were initiated with control eggs. If these levels of RDX were toxic, it should have been evident in the first generation exposure.

Eggs produced per adult per treatment level during this generation of the exposure are presented in Table 20. Since egg production had just initiated after 34 days exposure (alloted exposure period), egg collection continued to day 36.

No significant differences were observed for the parameters meausred between the negative control vessels and the positive - DMSO control vessels and they were therefore combined.

(G) Chronic Toxicity To Fathead Minnows - The results of analyses of water quality parameters measured throughout the chronic exposure indicated that hardness, alkalinity, acidity, dissolved oxygen and pH varied minimally between the various exposure aquaria and were not effected by treatment concentrations of RDX. The mean measured concentrations of RDX in water during both chronic exposures are presented in Table 22.

During the first chronic exposure, concentrations of RDX as high as 4.9 mg/l had no significant effect on the percentage of fry hatching or on fry survival and total lengths during the initial

30 days of exposure (Table 23). After 60 days, survival of fry exposed to 4.9 mg/l RDX was lower than survival of fry controls and lesser treatment concentrations. Total lengths of fathead minnows exposed 60 days remained uneffected by concentrations of RDX $\leq$4.9 mg/l. The first tests ended after 140 days of exposure when all fish were accidently killed by an excessive dosage of formalin-malachite green which was administered as a therapeutic treatment for external parasites. Between test days 63 and 140, no mortality was observed in any of the exposure aquaria and fish were developing normally.

Due to the problem in the previous chronic exposure, a second test was initiated to determine the chronic effects of RDX. As in the first test, percentage hatchability of fathead minnow eggs was not significantly effected by concentrations of RDX $\leq$6.3 mg/l (Table 24). After 30 days, survival of fry exposed to 6.3 mg/l RDX was significantly lower than control, and was further reduced after 60 days exposure. Mortality of fry exposed to 6.3 mg/l began earlier and was more extensive than had been observed in the previous test. This was most likely due to the measured concentration of RDX in the high treatment level (6.3 mg/l) of the second chronic being higher than that in the first chronic test (4.9 mg/l). Total lengths of fathead minnows after 30 and 60 days were uneffected by exposure to concentrations of RDX as high as 6.3 mg/l.

Between test days 64 and 181, survival of fathead minnows was not effected by exposure to RDX, with only four mortalities observed during the entire period. This compares closely with observations from the previous chronic test to suggest that RDX is not cumulatively toxic after the initial 60 days of exposure. During the spawning period, mortality of several male fish occurred in individual replicates of the control, solvent control and 0.43 mg/l RDX treatments (Table 25). In view of the absence of mortality in the higher RDX treatments, these mortalities were probably the result of stress during spawning. After 240 days exposure, total lengths and wet weights of mature male and female fathead minnows were not effected by exposure to concentrations of RDX as high as 6.3 mg/l.

The data presented in Table 26 indicate that a similar level of spawning activity occurred in all tanks during the test. No significant differences were found in the total number of spawns, total number of eggs or the mean number of eggs per spawn which were produced in controls and concentrations of RDX $\leq$ 6.3 mg/l. Due to the early mortality of fish exposed to 6.3 mg/l, fewer females had survived to the spawning period. As a result, the number of spawns per female and the number of eggs per female were significantly higher in this concentration than in controls and lesser concentrations of RDX. The mean percentage hatchability was similar for eggs spawned by control fish and those spawned by minnows exposed to concentrations of RDX as high as 6.3 mg/l.

There were no discernible effects of RDX on survival or growth of second generation fathead minnows after 30 days exposure to concentrations as high as 6.3 mg/l (Table 27). These data compare favorably with results of the critical life stage study and the first chronic test, but are in conflict with reduced survival of first generation fish observed after 30 days exposure to 6.3 mg/l during the second chronic.

DISCUSSION AND CRITERIA FORMULATION

The range of static and incipient LC50 values determined during the static and dynamic toxicity tests are very similar for all species of fish, while the LC50 values to be determined for invertebrate species remained greater than the highest levels tested. The data from the critical life stage studies indicate an effect on growth at the highest concentration (5.8 mg/l) tested. No toxicant related effects were observed among midges tested chronically up to a concentration of 21 mg/l. The production of young daphnids per parthenogenetic female was significantly reduced on days 7 and 14 of the chronic exposure. This may have been due to a higher concentration of RDX in solution at these time intervals, thereby inhibiting production of young. During the initial 30 days of the first fathead minnow chronic, no effect was observed, while during the initial 30 days of the second fathead minnow chronic, the high concentration (6.3 mg/l) exhibited a significant decrease in survival. Both exposures exhibited significant decreases in survival at 60 days of exposure. These differences are most likely due to the higher concentration of RDX in solution during the second chronic test. Survival of fathead minnows between test days 64 and 181 was not effected during either chronic study. In addition, a similar level of spawning activity occurred in all tanks during the test.

The above data indicate, with the exception of the daphnid information, that the toxic action of RDX is probably due to the acute toxicity of this compound. The LC50's for fish static and dynamic toxicity tests ranged from 3.6 to 8.4 mg/l, and all of the observed toxicity for the above tests was within this range. In fact, the incipient LC50 for fathead minnows exposed to RDX for 11 days was determined to be 5.2 mg/l. This is close to the high concentrations of both fathead minnow chronic studies, and appears to explain the initial mortality. Additionally, the data on bioconcentrations of 14C-RDX indicate that there is little propensity for RDX to accumulate in either muscle of visceral portions of any of the fishes tested.

The generally accepted expression for that concentration of a water quality constituent reasonably judged to preclude hazard to aquatic organisms due to that presence of that constituent is a water quality criterion.

In general, water quality criteria specify concentrations of water constituents which when not exceeded will protect one organism, an organism community, or a prescribed water quality use with an adequate margin of safety during continuous chronic exposure. The prcceudres for genesis of valid water quality criteria have been well defined (National Technical Advisory Committee, 1968; National Academy of Sciences, National Academy of Engineering, 1972; U.S. Environmental Protection Agency, 1976). Water quality criteria reflect a knowledge of both environmental

accumulation and persistence, and acute and chronic toxicity of specific toxicants in aquatic ecosystems. The most critical aspect in formulating a valid water quality criterion is in developing an understanding of the relationship between the acute and chronic toxicity of a chemical to aquatic organisms.

Based on the broad spectrum of acute toxicity data, information of the accumulation of RDX residues in biological systems, a clear understanding of the relationship between the acute and chronic toxicity of RDX to aquatic organisms, and a reasonable data base which supports the applicability of this relationship to broad taxonomic groups, we believe these research efforts provide a sound data base for the formulation of a valid water quality criteria for RDX for the protection of freshwater aquatic life with an adequate margin of safety.

Results of static acute toxicity tests with a wide variety of freshwater aquatic organisms representing several trophic forms under a variety of water quality conditions indicates that the LC50 values generally are greater than 3 mg/l RDX (Tables 2, 5, 6, and 7). The data from the bioconcentration study utilizing 14C-RDX and several species of fish clearly indicates that RDX has a relatively low bioconcentration potential (Tables 10, 11, 12 and Figures 2 and 4). Thus, the critical aspect of a criterion formulation is accurately defining the specific empirically-derived application factor for RDX which should be utilized to estimate the chronic toxicity of this chemical to aquatic organisms based

on available acute toxicity data.

We have determined during flow-through acute toxicity tests that the 96-hour LC50 of RDX to fathead minnows is 5.2 mg/l. We have further determined during chronic toxicity studies with fathead minnows that the chronically safe concentrations based on toxic effects over one complete life cycle is between 3.0 and 6.3 mg/l. Thus, based on an understanding of the chronic toxicity of RDX to fathead minnows, the limits of the empirically defined ratio (application factor) specific for RDX are >3.0/5.2 and <6.3/5.2 or >0 58 <1.2.

In view of these data, it appears that applying an application factor of 0.1 to the acute toxicity data would adequately protect aquatic life during chronic exposure. Applying this application factor to the lowest limit of the observed acute toxicity values for freshwater organisms (3. 6 mg/l, 96-hour LC50 for bluegill at pH 6.0, 20°C), we propose that a concentration of 0.35 mg/l RDX would be a reasonable water quality criterion providing for the protection of freshwater life with an ample margin of safety.

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Table 1 -- Physical characteristics of cyclotrimethylene trinitramine (RDX) evaluated in bioassays conducted at E G & G, Bionomics. All of the RDX shipments were under water (except the Sept. 10, 1974 and May 1, 1975 shipment).

Shippers	Physical characteristics	Date received
Holston Army Ammunition Plant (AAP) Kingsport, TN	white powder	September 10, 1974
Aberdeen Proving Ground Aberdeen, MD	white slurry	October 15, 1974
Holston AAP	white slurry	November 21, 1974
Holston AAP	white powder (standard)	May 1, 1975
Holston AAP	white slurry	May 19, 1975

Table 1 -- Acute toxicity values[a] (mg/l) for RDX utilizing aquatic invertebrates and fishes determined during static toxicity tests.

Species	Hours of exposure 24	48	96
Daphnia magna (water flea)	>100	>100	-
Gammarus fasciatus (scud)	>100	>100	-
Asellus militaris (sowbug)	>100	>100	-
Chironomus tentans (midge)	>100	>100	-
Lepomis macrochirus (bluegill)	14 (12-17)[b]	8.5 (7.5-9.5)	6.0 (5.4-6.5)
Salmo gairdneri (rainbow trout)	9.4 (8.5-10)	7.0 (6.3-7.7)	6.4 (5.4-7.4)
Ictalurus punctatus (channel catfish)	7.5 (6.7-8.5)	6.0 (5.3-6.9)	4.1 (3.5-4.9)
Pimephales promelas (fathead minnow)	10 (7.4-14)	5.8 (6.9-12)	5.8 (4.7-7.2)

[a] Acute toxicity values are expressed as effective concentrations causing immobilization (EC50) after 24 and 48 hours for invertebrates and lethal concentrations (LC50) after 24, 48 and 96 hours for fishes.

[b] 95% confidence interval.

Table 3 -- Percent change[a] in the cell density[b] of Selenastrum capricornutum, Microcystis aeruginosa, Anabaena flos-aquae, and Navicula pelliculosa after 96 hours exposure to RDX.

Nominal RDX concentration (mg/l)	S. capricornutum	M. aeruginosa	A. flos-aquae	N. pelliculosa
0.32	-2	0	0	0
1.0	-5	-1	0	0
3.2	-17	0	0	-2
10	-23	-7	-4	-8
32	-38	-18	-14	-17

[a] Percent change is relative to control cultures.

[b] Determined by cell counts for all species except A. flos-aquae which was determined by optical density.

Table 4 -- Percent change[a] in the chlorophyll a content of Selenastrum capricornutum, Microcystis aeruginosa, Anabeana flos-aquae and Navicula pelliculosa after 96 hours exposure to RDX.

Nominal RDX concentration (mg/l)	S. capricornutum	M. aeruginosa	A. flos-aquae	N. pelliculosa
0.32	-3	0	0	0
1.0	-16	-3	0	-3
3.2	-17	-1	-3	0
10	-26	-11	-6	-9
32	-22	-21	-17	-23

[a] Percent change is relative to control cultures.

Table 5 -- Acute toxicity of RDX to selected life stages of fathead minnows (Pimephales promelas) as determined during static toxicity tests.

Life stage	LC50 (mg/l) 24-hour	48-hour	96-hour	144-hour
eggs	>100	>100	>100	>100
1-hour post hatch	>100	>100	43 (27-69)[a]	-[b]
7-days post hatch	>32	18 (13-24)	3.8 (3.0-5.0)	-
30-days post hatch	18 (13-24)	16 (13-19)	16 (13-19)	-
60-days post hatch	11 (6.1-21)	11 (5.9-21)	11 (5.9-21)	-

[a] 95% confidence interval.

[b] Tests with fry were 96 hours in duration.

Table 6 -- Acute toxicity of RDX to bluegill (Lepomis macrochirus) under varying conditions of water quality during static toxicity tests.

Temperature (°C)	pH	Hardness (mg/l $CaCO_3$)	96-hour LC50 (mg/l)
15	7.0	35	8.4(6.0-11)[a]
20	7.0	35	5.1(3.9-6.7)
25	7.0	35	4.1(3.0-5.6)
20	7.0	35	3.8(2.0-7.1)
20	7.0	100	5.3(4.1-6.8)
20	7.0	250	3.9(2.1-7.3)
20	6.0	35	3.6(1.9-6.6)
20	7.0	35	3.7(2.0-6.9)
20	8.0	35	3.9(2.1-7.3)

[a] 95% confidence interval.

Table 7 -- Acute toxicity of aged solutions of RDX to bluegill (Lepomis macrochirus) during static toxicity tests.

Age of solutions prior to bioassay (hrs.)	96-hour LC50 (mg/l)
0	4.7(3.4-6.5)[a]
12	4.8(3.5-6.8)
24	5.1(3.6-7.2)
48	4.8(3.5-6.7)
96	4.8(3.5-6.7)

[a] 95% confidence interval.

Table 8 -- Acute toxicity of RDX to aquatic invertebrates and fishes during dynamic toxicity tests (nominal concentration).

Species	LC50 (mg/l) 24-hour	48-hour	96-hour	Incipient[a]
Daphnia magna (water flea)	>15	>15	-	>15
Chironomus tentans (midge)	>15	>15	-	>15
Lepomis macrochirus (bluegill)	>10		7.6 (5.6-10)[b]	6.4 (5.3-7.8)
Ictalurus punctatus (channel catfish)	>10		13 (8.8-20)	11 (9.1-13)
Pimephales promelas (fathead minnow)	>10		6.6 (5.0-8.7)	5 2 (4.3-6.4)

a
Incipient LC50 estimated after 96 hours for water flea, 96 hours for midge, 264 hours for bluegill, 264 hours for catfish, and 264 hours for minnows.

b
95% confidence interval.

Table 9 -- Mean measured 14C-residues calculated as RDX, in water during 28 days continuous exposure of bluegill (Lepomis macrochirus), channel catfish (Ictalurus punctatus) and fathead minnow (Pimephales promelas) to nominal concentrations of 0.010 and 1.0 mg/l 14C-RDX.

Day	Measured concentrations (mg/l) 0.010 mg/l	1.0 mg/l
0	0.0087 (0.0001)[a]	1.0 (0.1)
1	0.0098 (0.0001)	1.0 (0.1)
3	0.011 (0.001)	1.0 (0.1)
7	0.018 (0.002)[b]	1.0 (0.1)
10	0.032 (0.001)	1.0 (0.1)
14	0.011 (0.001)	0.37 (0.01)[c]
21	0.0096 (0.0001)	1.1 (0.1)
28	0.010 (0.001)	1.1 (0.1)

[a] Mean and standard deviation.

[b] High concentration due to system malfunction.

[c] Decreased value due to temporary system malfunction, datum not used $\bar{X} = 0.014 \pm 0.008$ $\bar{X} = 1.0 \pm 0.1$ calculations.

Table 10 -- Measured 14C-residues calculated as RDX in the edible and visceral tissue of bluegill (Lepomis macrochirus) during 28 days of continuous aqueous exposure to 14C-RDX concentrations of 0.014 and 1.0 mg/l, and during 14 days depuration.

		Mean measured 14C-residues (mg/kg)									
		0.014 mg/l					1.0 mg/l				
Period	Day	edible tissue		X[a]	viscera	X[b]	edible tissue		X[a]	viscera	X[b]
Exposure	1	0.029	(0.017)[c]	3.1	0.072 (0.059)	8	2.9	(1.3)	2.9	8.1 (4.1)	8
	3	0.035	(0.011)	3.5	0.095 (0.047)	9.5	4.3	(2.2)	4.3	13 (7)	13
	7	0.038	(0.016)	2.5	0.074 (0.048)	5	4.0	(1.8)	4.0	12 (5)	12
	10	0.048	(0.010)	1.9	0.04 (0.011)	2	4.7	(2.9)	4.7	12 (10)	12
	14	0.14	(0.01)	6.4	0.27 (0.04)	12	2.6	(1.5)	3.8	7.8 (4.1)	11
	21	0.048	(0.007)	4.8	0.13 (0.06)	10	3.0	(0.3)	4.1	7.5 (0.3)	10
	28	0.046	(0.005)	4.7	0.091 (0.014)	9	3.8	(0.1)	3.5	6.8 (1.1)	6
Depuration	1	0.040	(0.003)		0.067 (0.019)		3.1	(1.4)		8.4 (4.8)	
	3	0.049	(0.022)		0.083 (0.023)		3.0	(0.8)		3.5 -	
	7	0.033	(0.007)		0.064 (0.002)		1.5	(0.4)		2.7 (0.6)	
	10	0.031	(0.010)		0.034 (0.007)		2.0	(1.5)		4.0 (3.8)	
	14	<0.001			<0.002		3.6	(1.5)		6.4 (3.6)	

[a]Bioconcentration factor in edible tissue based on the mean concentration of 14C-RDX in water of the previous day and that day.

[b]Bioconcentration factor in non-edible tissue based on the mean concentration of 14C-RDX in water of the previous day and that day.

[c]Mean and standard deviation.

Table 11 -- Measured 14C-residues calculated as RDX, in the edible and visceral tissue of channel catfish (Ictalurus punctatus) during 28 days of continuous aqueous exposure to 14C-RDX concentrations of 0.014 and 1.0 mg/l, and during 14 days depuration.

Period	Day	Mean measured 14C-residues (mg/kg) 0.014 mg/l edible tissue	X[a]	viscera	X[b]	1.0 mg/l edible tissue	X[a]	viscera	X[b]
Exposure	1	0.016 (0.002)[c]	1.7	0.027 (0.006)	2.9	1.7 (0.1)	1.7	2.8 (0.3)	2.8
	3	0.019 (0.003)	1.9	0.028 (0.002)	2.8	1.7 (0.3)	1.7	2.9 (0.5)	2.9
	7	0.033 (0.004)	2.0	0.045 (0.002)	3	1.8 (0.1)	1.8	3.2 (0.6)	3.2
	10	0.071 (0.008)	2.8	0.12 (0.02)	4.8	2.0 (0.3)	2.0	3.2 (0.2)	3.2
	14	0.12 (0.01)	5.5	0.12 (0.11)	5.5	0.08 (0.2)	1.2	1.6 (0.4)	2.3
	21	0.032 (0.004)	3.2	0.060 (0.005)	6	2.5 (0.3)	3.4	4.1 (1.1)	5.5
	28	0.039 (0.003)	4.0	0.049 (0.008)	5	3.2 (0.1)	2.9	3.6 (0.2)	3.3
Depuration	1	0.020 (0.002)		0.030 (0.006)		0.96 (0.19)		1.8 (0.3)	
	3	0.017 (0.001)		0.024 (0.006)		0.78 (0.15)		1.0 (0.4)	
	7	0.017 (0.004)		0.026 (0.006)		0.65 (0.16)		0.78 (0.15)	
	10	0.013 (0.002)		0.015 (0.002)		0.66 (0.06)		0.74 (0.08)	
	14	<0.002		<0.002		0.56 (0.12)		0.47 (0.27)	

[a] Bioconcentration factor in edible tissue based on the mean concentration of 14C-RDX in water of the previous day and that day.

[b] Bioconcentration factor in non-edible tissue based on the mean concentration of 14C-RDX in water of the previous day and that day.

[c] Mean and standard deviation.

Table 12 -- Measured 14C-residues calculated as RDX, in the edible and visceral tissue of fathead minnow (Pimephales promelas) during 28 days of continuous aqueous exposure to 14C-RDX concentrations of 0.014 and 1.0 mg/l, and during 14 days of depuration.

		Mean measured 14C-residues (mg/kg)					
		0.014 mg/l			1.0 mg/l		
Period	Day	edible tissue	X^a	viscera	X^b	edible tissue	X^a	viscera	X^b
Exposure	1	0.013 (0.003)[c]	1.4	0.023 (0.002)	2.5	1.4 (0.2)	1.4	2.2 (0.2)	2.2
	3	0.018 (0.004)	1.8	0.033 (0.003)	3.3	2.0 (0.1)	2.0	3.8 (0.5)	3.8
	7	0.031 (0.008)	2.1	0.047 (0.005)	3.1	1.8 (0.2)	1.8	3.4 (0.1)	3.4
	10	0.074 (0.004)	3.0	0.15 (0.01)	6	2.1 (0.3)	2.1	4.1 (0.6)	4.1
	14	0.13 (0.01)	5.9	0.26 (0.03)	12	1.4 (0.5)	3.8	3.2 (0.8)	4.6
	21	0.047 (0.007)	4.7	0.047 (0.011)	10	3.1 (0.7)	4.2	7.7 (0.3)	10
	28	0.058 (0.012)	5.9	0.11 (0.02)	11	4.4 (0.4)	4.0	9.6 (0.7)	8.8
Depuration	1	0.037 (0.011)		0.070 (0.006)		2.2 (1.2)		4.5 (0.1)	
	3	0.023 (0.004)		0.071 (0.043)		2.1 (0.4)		3.8 (0.4)	
	7	0.028 (0.012)		0.050 (0.005)		1.8 (0.5)		3.4 (0.6)	
	10	0.029 (0.008)		0.038 (0.005)		2.2 (1.0)		3.2 (0.3)	
	14	0.029 (0.007)		0.032 (0.006)		1.3 (0.9)		1.6 (0.5)	

[a] Bioconcentration factor in edible tissue based on the mean concentration of 14C-RDX in water of the previous day and that day.

[b] Bioconcentration factor in non-edible tissue based on the mean concentration of 14C-RDX in water of the previous day and that day.

[c] Mean and standard deviation.

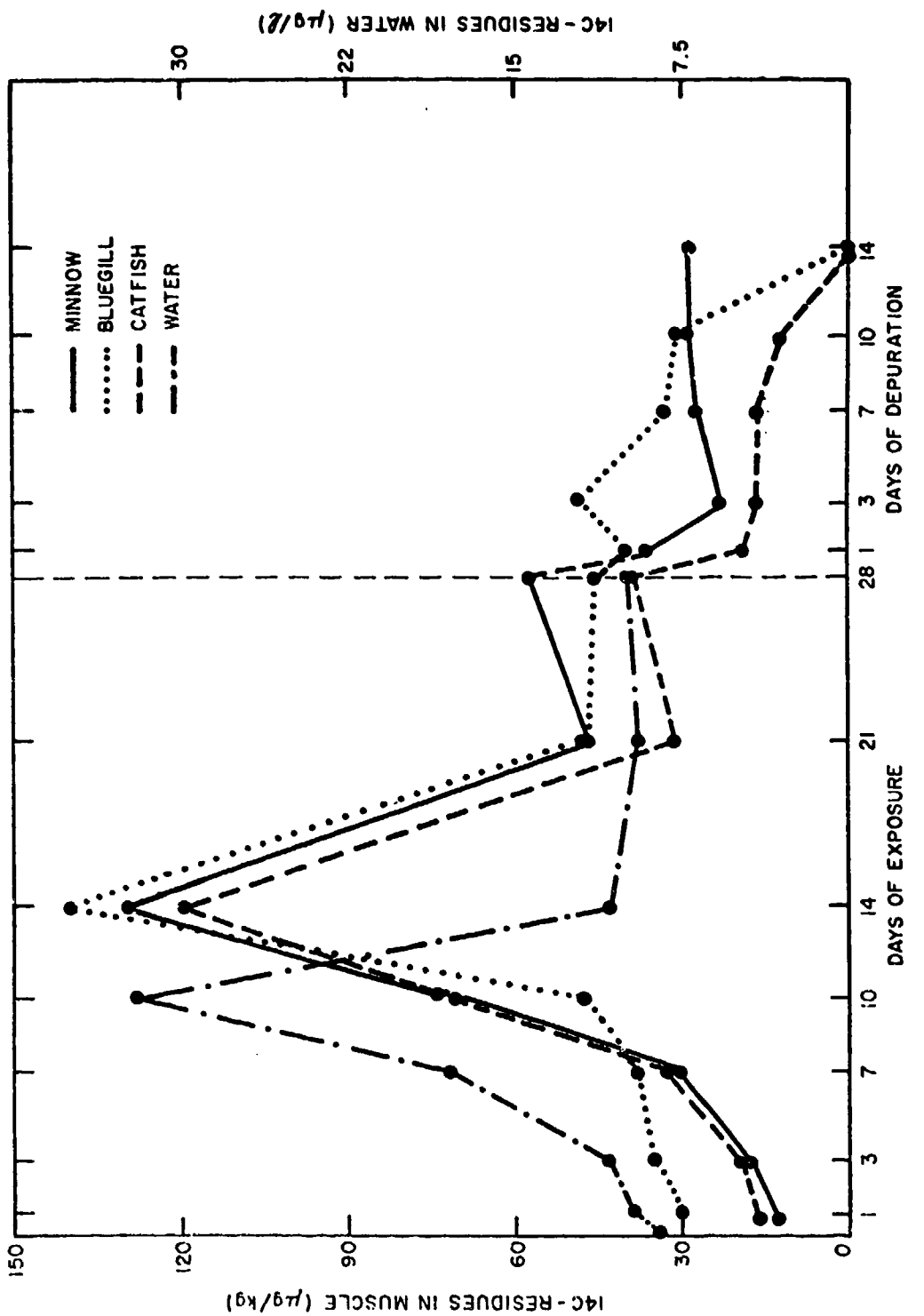
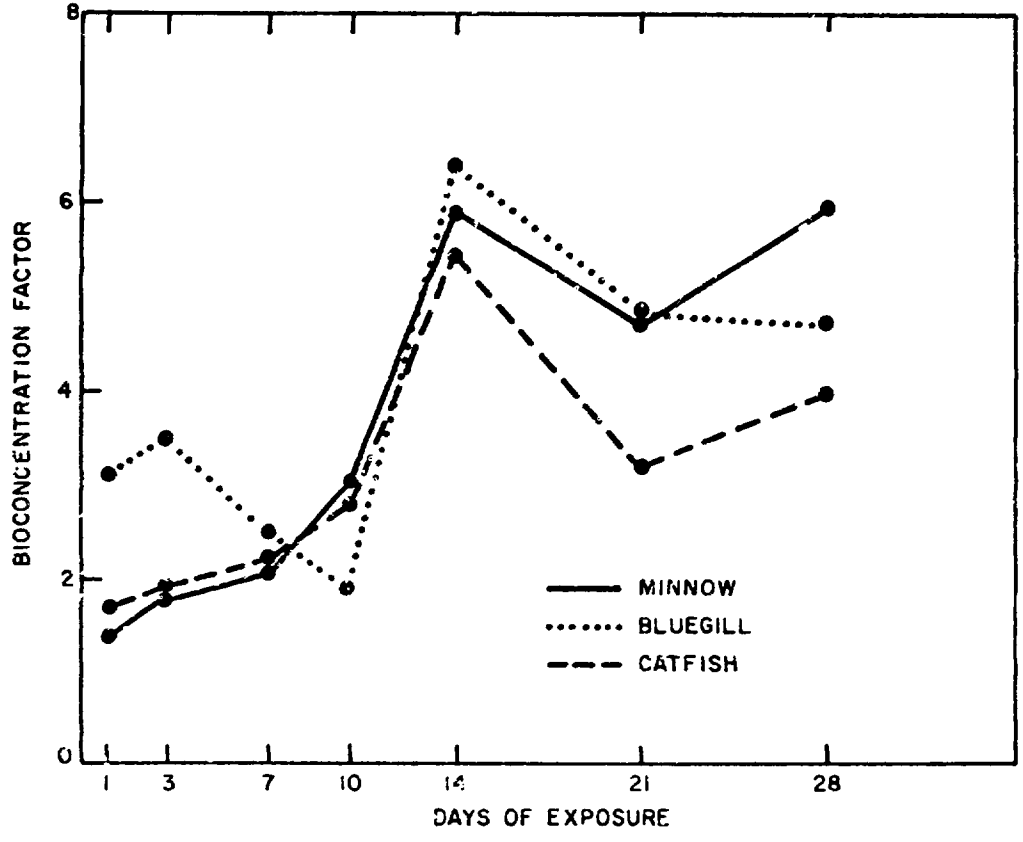


Figure 1. Mean measured 14C-residues, calculated as RDX, in the water and in fish muscle during 28 days continuous aqueous exposure to a nominal 14C-RDX concentration of 0.010 mg/l and during 14 days depuration in flowing, uncontaminated water.

Figure 2. Bioconcentration factors* during 28 days of continuous aqueous exposure of fish to a nominal ^{14}C-RDX concentration of 0.010 mg/l.



*Derived by dividing the measured ^{14}C-residues present in the muscle at any sample by the mean of the ^{14}C-residue concentrations in the water on that day and on the previous sample day.

Figure 3. Mean measured 14C-residues, calculated as RDX, in water and fish muscle during 28 days continuous aqueous exposure to a nominal 14C-RDX concentration of 1.0 mg/l and during 14 days depuration in flowing, uncontaminated water.

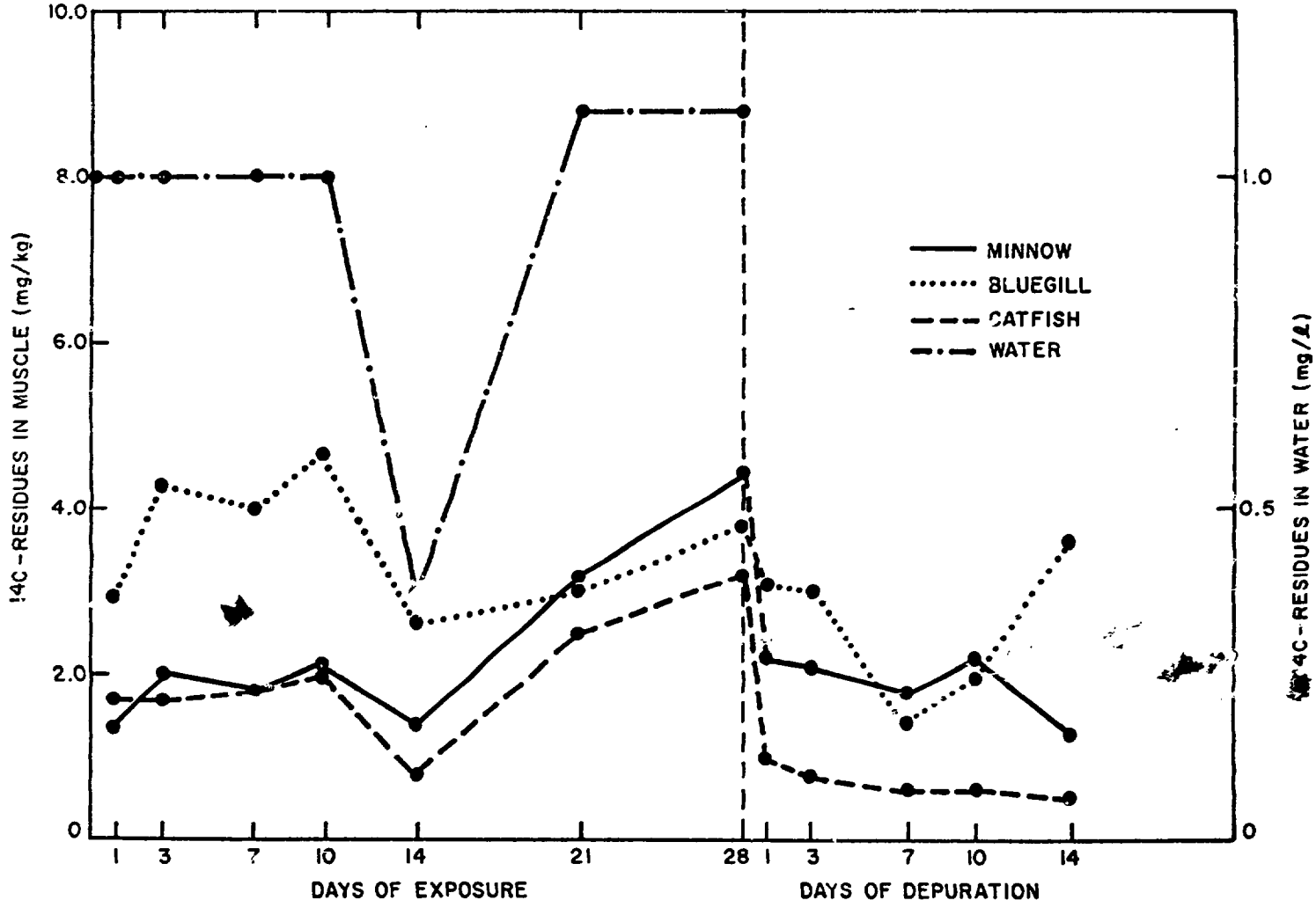
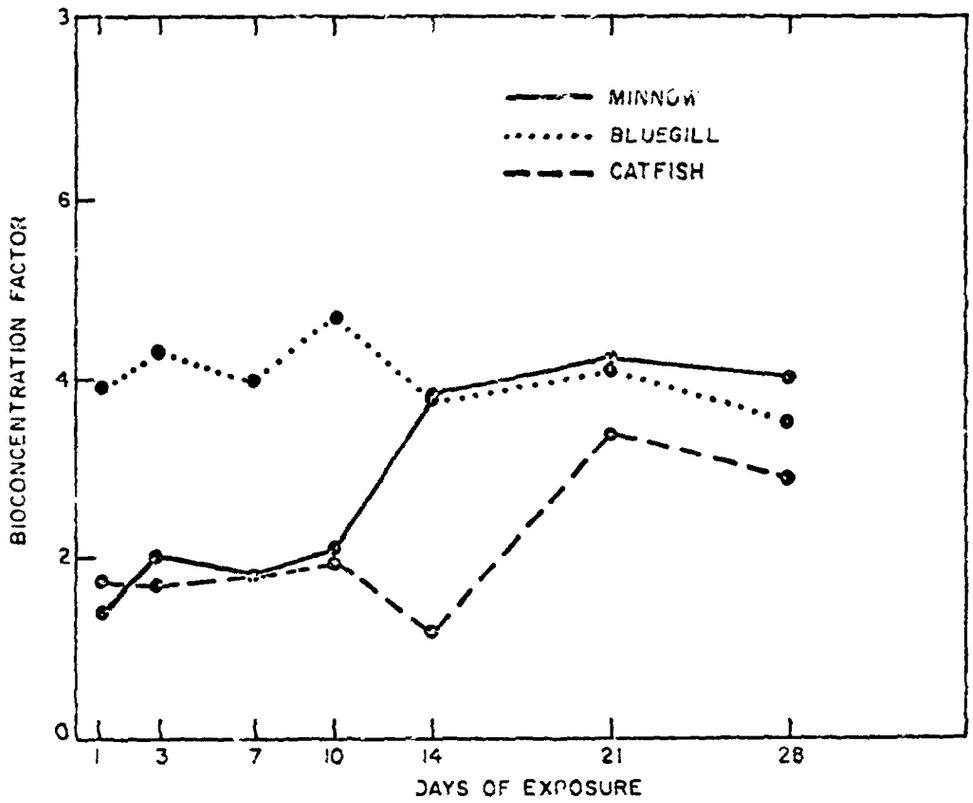


Figure 4. Bioconcentration factors* during 28 days of continuous aqueous exposure of fish to a nominal 14C-RDX concentration of 1.0 mg/l.



*Derived by dividing the measured 14C-residues present in the muscle at any sample by the mean of the 14C-residue concentrations in the water on that day and on the previous sample day.

Table 13 - Mean and standard deviation measured concentrations of RDX in water during egg and fry exposures of channel catfish and fathead minnow.

Nominal concentration (mg/l)	n	Mean measured concentration (mg/l)
Channel catfish		
2.0	5	2.3 ± 0.36
0.5	5	0.71 ± 0.17
0.125	5	0.11 ± 0.03
Fathead minnow		
5.0	2	5.8 ± 0.49
1.25	2	1.2 ± 0.56
0.13	2	0.26 ± 0.02

Table 14 -- Mean percentage hatch, mean percentage survival and total length of channel catfish (Ictalurus punctatus) continuously exposed to RDX.

measured concentration (mg/l)		Mean hatch (%)	30 days survival (%)	30 days mean total length (mm)
2.3	A	93	26[a,b]	24 ± 3
	B	100	44	23 ± 2
1.2	A	83	36[a,b]	23 ± 2
	B	94	38	24 ± 2
0.71	A	88	100	23 ± 2
	B	98	100	23 ± 2
0.30	A	96	96	24 ± 2
	B	100	100	23 ± 4
0.11	A	97	96	23 ± 2
	B	83	98	23 ± 2
control	A	96	98	23 ± 2
	B	96	96	23 ± 2
solvent control	A	96	100	23 ± 2
	B	97	98	24 ± 2

[a] May be due to diluter malfunction resulting in higher than nominal concentrations of RDX.

[b] $F=39.68$ $F_{0.05}(6,6)=4.28$; $d'=15.20$

Table 15 -- Mean percentage hatch, mean percentage survival and total length of fathead minnow (Pimephales promelas) continuously exposed to RDX.

measured concentration (mg/l)		Mean hatch (%)	30 days survival (%)	30 days total length (mm)
5.8	A	78	87.5	17 ± 3[a]
	B	66	85	17 ± 3
3.0	A	64	82.5	18 ± 3
	B	69	87.5	18 ± 3
1.2	A	71	92.5	19 ± 3
	B	78	92.5	19 ± 3
0.76	A	69	67.5	19 ± 4
	B	59	70	20 ± 3
0.26	A	79	100	19 ± 3
	B	56	80	19 ± 3
control	A	63	95	18 ± 3
	B	75	85	19 ± 2
solvent control	A	80	92.5	19 ± 3
	B	68	100	19 ± 3

[a] $F=11.8$, $F=0.05$, $(6,6)=4.28$; $d'=1.01$

Table 16 - Mean and standard deviation measured concentrations of RDX in water during chronic exposures of Daphnia magna and Chironomus tentans.

Nominal concentration (mg/l)	n	Mean measured concentration (mg/l)
D. magna		
15	4	20 ± 3.1
7.5	4	9.5 ± 1.5
3.7	4	4.8 ± 1.2
1.8	4	2.2 ± 0.35
0.90	4	1.4 ± 0.44
C. tentans		
15	5	21 ± 3.6
7.5	6	10 ± 6.7
3.7	6	4.0 ± 0.75
1.8	6	2.2 ± 0.48
0.90	5	1.3 ± 0.21

Table 17 -- Weekly mean (standard deviation) percent survival of the water flea (Daphnia magna) exposed to RDX up to 42 days.

measured concentration (mg/l)	Mean percent survival[a]					
	Generation 1			Generation 2		
	day/ 7	14	21	28	35	42
control	79(11)	78(12)	78(12)	82(13)	76(20)	76(20)
1.4	77(26)	78(26)	78(26)	86(14)	58(31)	58(28)
2.2	81(22)	81(22)	75(30)	92(9)	68(32)	62(35)
4.8	95(7)	76(35)	72(	91(12)	82(16)	74(22)
9.5	81(15)	80(15)	71(	92(15)	70(34)	69(34)
20	90(12)	86(11)	71(21)	95(10)	91(8)	90(8)

[a] Each survival value represents the mean of 4 replicate treatment vessels.

Table 18 -- Mean (standard deviation) number of young water flea produced per parthenogenetic female Daphnia magna exposed to RDX during the 42 day exposure.

measured concentration (mg/l)	Mean young produced/parthenogentic female[a]			
	Generation 1		Generation 2	
day/	14	21	35	42
controls	22(12)	14(4)	28(2)	14(6)
1.4	17(8)	10(1)	8(4)	13(5)
2.2	22(11)	13(4)	14(6)	17(11)
4.8	6(4)[b]	9(4)	9(4)	14(5)
9.5	5(2)[b]	16(6)	14(8)	21(11)
20	3(1)[b]	10(4)	10(4)	14(1)

[a] Each value represents the mean of 4 replicate treatment vessels.

[b] $F=4.86$, $F_{0.05}(5,15)=2.90$; $d'=14.0$

Table 19 -- Mean (standard deviation) percent survival of the F_o midges (Chironomus tentans) larvae[a], pupae[b], adults[b] and percent emergence of adults[b] after continuous exposure to RDX.

measured concentration (mg/l)	Percent survival[c]			Percent emergence[c]
	larvae	pupae	adults	
controls	41(15)	97(2)	76(17)	86(24)
1.3	33(16)	88(12)	58(24)	76(26)
2.2	38(8)	98(2)	98(3)	86(16)
4.0	33(9)	89(19)	81(32)	63(44)
10	22(15)[d]	92(10)	41(41)	67(8)
21	28(13)	95(10)	80(18)	59(29)

[a] Larvae survival determined after 14 days of exposure.

[b] Pupae, adult survival, percent emergence determined after 23 days of exposure.

[c] Each value represents the mean of four replicate treatment vessels.

[d] $F=3.35$, $F0.05\ (5,15)=2.90$; $d'=15.8$

Table 20 -- Total eggs produced per adult Chironomus tentans during the two generation exposure to RDX.

measured concentration (mg/l)	Egg/adult/treatment[a]	
	generation 1[b]	generation 2[c]
control	35	17
1.3	12[d]	17
2.2	8	9
4.0	15[d]	38
10	0[e]	15
21	14	22

[a] Each value represents the sumation of four replicate treatment vessels.

[b] Eggs produced after 27 days exposure.

[c] Eggs produced after 36 days exposure.

[d] None of the eggs obtained were fertile. Second generation initiated with control eggs.

[e] Second generation initiated with control eggs.

Table 21 -- Mean (standard deviation) percent survival of the F_1 midges (Chironomus tentans) larvae[a], pupae[b], adults[b] and percent emergence of adults[b] after continuous exposure to RDX.

measured concentration (mg/l)	Percent survival[c]			Percent emergence[c]
	larvae	pupae	adults	
control	52(18)	89(9)	85(8)	92(9)
1.3[d]	30(22)[e]	72(23)	33(2)[f]	100(0)
2.2	36(4)[e]	95(4)	70(21)	50(10)[g]
4.0[d]	25(11)[e]	87(4)	53(33)[f]	92(7)
10[d]	38(7)[e]	90(8)	78(12)	92(10)
21	38(12)[e]	89(11)	67(13)	77(33)

[a] Larvae survival determined after 19 days of exposure.

[b] Pupae and adult survival and percent emergence determined after 34 days of exposure.

[c] Each value represents the mean of four replicates.

[d] Second generation exposure initiated with control eggs.

[e] $F=3.48$, $F_{0.05}(5,15)=2.90$; $d'=11.1$

[f] $F=3.03$, $F_{0.05}(5,15)=2.90$; $d'=24.1$

[g] $F=4.73$, $F_{0.05}(5,15)=2.90$; $d'=30.2$

Table 22 - Mean and standard deviation of measured concentrations of RDX in water during chronic exposures of fathead minnows.

Nominal concentration (mg/l)		n	Mean measured concentration (mg/l
Test #1	5.0	4	4.9 ± 0.41
	2.5	4	2.7 ± 0.39
	1.3	4	1.1 ± 0.20
	0.63	4	0.64 ± 0.08
	0.31	4	0.29 ± 0.02
Test #2	5.0	12	6.3 ± 2.2
	2.5	12	3.0 ± 0.82
	1.3	12	1.5 ± 0.38
	0.63	12	0.78 ± 0.27
	0.31	12	0.43 ± 0.11
	Control	2	<0.027

Table 23 -- Effects on first generation fathead minnows (Pimephales promelas) during continuous exposure to RDX (test 1)[a].

Mean measured concentration (mg/l)		Hatch (%)	30 days survival (%)	30 days total length (mm)	60 days survival (%)	60 days total length (mm)	60 days wet weight (g)
4.9	A	70	83	20 ± 3	75	29 ± 6	0.14
	B	88	93	18 ± 4	65	27 ± 5	0.13
2.7	A	70	100	19 ± 3	100	28 ± 3	0.18
	B	70	98	19 ± 2	98	27 ± 3	0.15
1.1	A	70	95	21 ± 3	95	31 ± 3	0.21
	B	78	95	19 ± 2	95	30 ± 3	0.19
0.64	A	72	100	19 ± 2	100	28 ± 4	0.16
	B	83	95	20 ± 2	95	29 ± 4	0.24
0.29	A	95	100	20 ± 2	100	29 ± 4	0.21
	B	76	95	20 ± 2	95	30 ± 2	0.22
solvent control	A	73	98	20 ± 3	98	28 ± 5	0.19
	B	77	100	19 ± 2	98	27 ± 4	0.21
control	A	88	93	20 ± 2	93	29 ± 4	0.21
	B	88	100	19 ± 2	100	26 ± 4	0.17

[a] Test ended on day 140 when all fish were accidentally killed by treatment for external parasites.

Table 24 -- Effects on first generation fathead minnows (Pimephales promelas) during continuous exposure to RDX (Test II).

Measured concentration (mg/l)		Hatch (%)	30 days survival (%)	30 days total length (mm)	60 days survival (%)	60 days total length (mm)
6.3	A	92	25[a]	22 ± 3	15[b]	33 ± 6
	B	93	28	21 ± 2	15	33 ± 2
3.0	A	97	98	20 ± 4	98	30 ± 5
	B	100	98	20 ± 3	93	30 ± 4
1.5	A	95	93	21 ± 3	93	32 ± 5
	B	97	95	21 ± 3	95	31 ± 5
0.78	A	92	90	21 ± 3	90	31 ± 4
	B	92	100	20 ± 3	100	31 ± 4
0.43	A	97	90	21 ± 3	90	31 ± 3
	B	95	98	21 ± 3	98	30 ± 4
solvent control	A	95	93	21 ± 3	93	32 ± 5
	B	92	93	20 ± 3	93	31 ± 3
control	A	97	95	21 ± 3	95	30 ± 3
	B	95	100	20 ± 4	100	29 ± 6

[a] $F=26.23$, $F_{0.05}(6,6)=4.28$; $d'=19.12$

[b] $F=23.36$, $F_{0.05}(6,6)=4.28$; $d'=23.25$

Table 25 -- Survival, sexual maturity, and growth of fathead minnows exposed 240 days to RDX.

Measured concentration (mg/l)		Number of survivors: mature males	Number of survivors: mature females	Total length (mm): males	Total length (mm): females	Wet weight (g): males	Wet weight (g): females
6.3	A	2	2	61	56	3.00	1.73
	B	3	3	62	53	3.46	1.67
3.0	A	4	6	64	50	3.33	1.45
	B	4	4	60	50	3.07	1.52
1.5	A	3	5	63	51	3.05	1.52
	B	3	7	65	50	3.27	1.42
0.78	A	3	7	64	51	3.57	1.51
	B	4	6	65	52	3.60	1.62
0.43	A	3	7	59	50	2.86	1.55
	B	2	6	57	50	3.16	1.48
solvent control	A	0[a]	7	-[a]	54	-[a]	1.92
	B	4	6	61	50	2.98	1.47
control	A	2	5	60	52	3.17	1.83
	B	3	7	63	48	3.73	1.38

[a] Three male fish died during the last 30 days of spawning.

Table 26 -- Egg production and hatchability of eggs from fathead minnows continuously exposed for 240 days to RDX.

Measured concentration (mg/l)		Total spawns	Total eggs	Eggs per spawn	Spawns per female	Eggs per female	Hatchability Mean ± S.D. (%)	Hatchability (N)[a]
6.3	A	30	6,183	206	15[b]	3,091[c]	85 ± 8.4	10
	B	43	6,994	163	14	2,331	95 ± 3.8	10
3.0	A	24	4,269	173	4	711	94 ± 6.9	10
	B	31	5,222	168	8	1,305	91 ± 7.9	10
1.5	A	28	5,182	185	6	1,036	84 ± 9.8	9
	B	38	6,645	175	5	949	93 ± 5.4	10
0.78	A	46	9,009	196	7	1,287	91 ± 6.4	10
	B	32	6,510	203	5	1,085	90 ± 8.2	9
0.43	A	37	8,236	223	5	1,177	89 ± 9.7	10
	B	39	8,333	214	6	1,389	90 ± 8.2	10
solvent control	A	29	6,044	208	4	863	87 ± 8.0	10
	B	27	4,404	163	5	734	91 ± 7.8	10
control	A	19	4,453	234	4	891	90 ± 7.1	9
	B	32	6,645	208	5	949	90 ± 10	10

[a] Number of egg groups used to determine mean hatch.

[b] $F=12.54$, $F_{0.05(6,6)}=4.28$; $d'=5.29$

[c] $F=10.00$, $F_{0.05\ (6,6)}=4.28$; $d'=1,098$

Table 27 -- Survival and growth of second generation (F_1) fathead minnows after 30 days continuous exposure to RDX.

Measured concentration (mg/l)		Survival (%)	Total length (mm) Mean ± S.D.	Wet weight (g)
6.3	A	85	24 ± 2	0.115
	B	98	23 ± 3	0.092
3.0	A	88	23 ± 3	0.126
	B	93	23 ± 2	0.109
1.5	A	83	23 ± 3	0.119
	B	73	24 ± 3	0.141
0.78	A	75	24 ± 3	0.133
	B	78	25 ± 3	0.141
0.43	A	90	24 ± 2	0.105
	B	90	24 ± 2	0.119
solvent control	A	75	23 ± 2	0.110
	B	95	24 ± 2	0.118
control	A	68	23 ± 2	0.112
	B	85	22 ± 2	0.091

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